SEP 13 1923

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

VOLUME XXXVIII, No. 3 SEPTEMBER 1, 1923

PUBLISHED MONTHLY

AT MOUNT ROYAL AND GUILFORD AVES., BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

COPYRIGHT, 1923, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3, 1879.
Accepted for mailing at special rate of postage provided for in section 1103, act of October 3, 1917.
Authorized June 29, 1918.

Made in United States of America

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

The Journal of Experimental Medicine is designed to cover the field of experimental medicine. Information regarding contributions and subscriptions is given in full on the back cover.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

EDITED BY

STANLEY R. BENEDICT, New York, N. Y. LAFAYETTE B. MENDEL, New Haven, Conn.
H. D. DAKIN, Scarborough, N. Y. DONALD D. VAN SLYKE, New York, N. Y.

The Journal of Biological Chemistry is designed for the prompt publication of original investigations of a chemical nature in the biological sciences. It is issued monthly. Each volume consists of as many numbers as are required to make a total of approximately 800 pages. The subscription price is $5.00 per volume to subscribers in the United States and Canada, payable in advance; $5.25 to those in other countries. The number of volumes issued yearly averages four. The price of single copies varies; it is given on the back cover of each number. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Biological Chemistry*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITED BY

JACQUES LOEB, New York, N. Y. W. J. V. OSTERHOUT, Cambridge, Mass.

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. It is issued bimonthly, one volume of about 600 pages appearing in a year. The subscription price per year (one volume) is $5.00, payable in advance. Single copies cost $1.00. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of General Physiology*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

STUDIES FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

The published results of investigations conducted in the Institute, or under its grants, are published at irregular intervals in volumes designated *Studies from The Rockefeller Institute for Medical Research*. The subscription price is $2.00 per volume, payable in advance. A special rate of $1.00 per volume is made to those who subscribe to *The Journal of Experimental Medicine*, *The Journal of Biological Chemistry*, or *The Journal of General Physiology*. Remittances should be made by draft or check on New York, or by postal money order, payable to The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.

SEMIANNUAL LIST

The Institute publishes a *Semiannual List* of all papers by members of the staff and those working under grants from the Institute, stating the title and place of publication of these reports. The *Semiannual List* will be sent regularly upon application.

WAVERLY PRESS
WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

LEISHMANIA DONOVANI IN THE PERIPHERAL BLOOD.

By CHARLES W. YOUNG, M.D., and HELEN M. VAN SANT.

(From the Department of Medicine of the Peking Union Medical College, Peking, China.)

(Received for publication, April 16, 1923.)

When Donovan (1) discovered Leishman-Donovan bodies in the spleen juice of a living patient he sent smears to Laveran and Mesnil, who named the organism *Piroplasma donovani*, because they thought it was inside the red blood cells (2). Stimulated by the work of these investigators Donovan found the parasite in smears of blood from the finger. 2 months after the appearance of the first communication Laveran (3) reported Cathoire's case, the first of so called infantile kala-azar from the Mediterranean area. He stated that Cathoire also found the organism in the blood of the greater circulation. In the same year (1904) Christophers (4) studied the histology of the tissues in kala-azar, and came to the conclusion that the disease is essentially a septicemia in which the organism is phagocytosed by the endothelial cells of the blood vessels and spleen; further, that the parasites multiply in these cells and, when the latter rupture, are freed in the blood, to be taken up by other similar cells. Knowles (5), on the contrary, says that "kala-azar is a disease which essentially involves the internal and vital organs of the body. . . . the presence of the L. D. body in the peripheral blood of the patient is a purely fortuitous phenomenon."

Rogers' first medium was merely citrated saline added to the spleen juice. Nicolle developed the N. N. N. medium[1] because he found results inconstant with the earlier method. Rogers (6) later observed that he was able to obtain only feeble or no growth in N. N. N. medium made with human blood. Cornwall and LaFrenais (7) made N. N. N. medium, using the blood of sheep, man, guinea pig, rabbit, goat, and dog. They obtained feeble growth on the goat blood medium, good growth on the rabbit blood, but none on the others, including that of man. They obtained subcultures on N. N. N. and nutrose media containing hydrocele fluid, however.

---

[1] The formula of the N. N. N. medium is as follows:

Agar	14 gm.
Salt	6 gm.
Water	900 cc.

The usual method of finding *Leishmania donovani* is the examination of stained smears or the culture of spleen juice obtained by aspiration with a needle. This latter procedure, however, is not devoid of danger (1, 8–11). Blood culture has been suggested as a substitute for spleen juice culture, but up to the present time this method has proved disappointing on account of inconstancy of results (12–15, 5, 7). Many have tried to use the presence of the parasite in the peripheral blood as a means of diagnosis (16–18, 5, 19).

In view of the somewhat contradictory results the following experiments were set up.

EXPERIMENTAL.

Washed red cells and serum were added separately to the slightly modified N. N. N. medium which, of course, did not contain the usual rabbit blood. The reaction was adjusted to pH 7.8 by the colorimetric method. Washed red cells and serum of the species indicated were added in the same proportion as in the ordinary N. N. N. medium (Table I).

TABLE I.

Effect of Red Cells and Serum from Different Species.

	Man.	Horse.	Sheep.	Rabbit.
Washed red cells.	−	−	−	+
	−	−	−	+
Serum.	−	−	Contaminated.	−
	−	−	"	−

The experiment was repeated in quadruplicate, with one loopful of culture for Tubes 1 and 2, and 1 drop for Tubes 3 and 4. In addition, the cells and serum were (*a*) heated at 56° C. for ½ hour, and (*b*) unheated. The medium had a hydrogen ion concentration of pH 7.6 (Table II). All tubes were examined after 12 days.

From these experiments it was evident that some specimens of human blood at least are unsuitable for the growth of *Leishmania* flagellates and that the serum is less favorable than the cells. As a result of the experiments it seemed desirable to remove both red cells and serum from the material to be planted. This was done by differential centrifugalization, after dilution at the time of obtaining the specimen. The procedure adopted was as follows:

10 cc. of blood were drawn from a convenient vein into a sterile syringe containing about 2 cc. of sterile citrated Locke's solution[2] and expelled into a flask containing from 50 to 70 cc. of the same solution. After mixing, this was taken to the laboratory and divided between two 50 cc. centrifuge tubes. It was centrifugalized at low speed[3] until the red cells were lightly packed at the

TABLE II.

Effect of Red Cells and Serum from Different Species.

	Tube No.	Man.	Horse.	Sheep.	Rabbit.
A	1	A few.	−	−	+
Red cells, heated.	2	" "	+	−	+
	3	+	+	±*	+
	4	A few.	+	±	+
B	1	" "	−	−	+
Red cells, unheated.	2	" "	−	+	+
	3	" "	−	+	++
	4	Contaminated.	Contaminated.	+	+
A	1	−	−	−	+
Serum, heated.	2	−	One flagellate seen.	−	+
	3	−	+	±	+
	4	−	−	±	+
B	1	−	−	−	+
Serum, unheated.	2	−	−	−	−
	3	−	−	−	+++
	4	−	+	±	+
Whole blood.	1	−	−	−	++++
	3	−	−	+	++++

* ± indicates that non-motile bodies were seen which resembled partly degenerated flagellates but could not be definitely identified as such.

[2] The formula for the citrated Locke's solution is as follows:

Sodium chloride	9.2 gm.
Potassium chloride	0.5 gm.
Calcium chloride	0.1 gm.
Sodium citrate	10.0 gm.
Distilled water	1,000.0 cc.

After autoclaving, the hydrogen ion concentration was about pH 7.4.

[3] 5 minutes at 750 revolutions per minute with a 20 cm. radius.

bottom, while the supernatant fluid was cloudy. The cloudy fluid was decanted or pipetted into another sterile tube and centrifugalized at high speed.[4] Stained smears of this sediment showed it to be composed almost entirely of blood platelets, with a few red cells and fewer polymorphonuclear leucocytes. On careful search extracellular Leishman-Donovan bodies have occasionally been found. No intracellular forms have ever been encountered. This sediment from the platelet fraction was planted into a series of tubes of modified N. N. N. medium[5] made with rabbit blood[6] with a reaction of about pH 7.6. The cultures were incubated at 22°C. At least 10 days usually elapsed before the flagellates were discoverable.

With this technique, 147 cultures were made from 34 specimens of blood from untreated cases of kala-azar and 161 cultures from 40 specimens of blood from treated cases. In some instances several specimens were taken from the same patient at intervals during the course of the disease. The net total was 308 cultures from 74 specimens from 42 patients. The results of these cultures are given in Tables III to V. They are divided into untreated cases (Table III) and treated cases (Tables IV and V). The treatment was estimated in milligrams of antimony[7] per kilo of patient's weight and the cultures were arranged in order of ascending total dosage on this basis. Table IV includes the treated cases up to the point where the unbroken succession of negative cultures begins. Table V shows the treated cases beyond that point. In the tabulation of cultures all

[4] 5 minutes at 1,375 revolutions per minute with a 20 cm. radius.

[5] Nicolle's formula was modified by adding 0.2 per cent dipotassium phosphate (K_2HPO_4) as a buffer.

[6] In other experiments not yet published the relation of the hydrogen ion concentration of the N. N. N. media to the growth of *Leishmania* was studied. Briefly, it may be said that, contrary to previous work, the organism was found to grow in media having a range from pH 5.0 to 9.8. At the acid end the growth was either negative or not abundant, but at the alkaline end there was no indication that the limit had been reached. For routine cultures a pH of 7.4 to 7.8 was used.

[7] The antimony preparations used for treatment were a 1 per cent solution of antimony potassium tartrate (tartar emetic); a solution of antimony sodium tartrate, usually 2 per cent; a 0.2 per cent suspension of colloidal antimony sulfide (Rogers, L., *Lancet*, 1919, i, 505); antimony silver arsaminol (Hata); and stibenyl. As the antimony content of each of these substances differs from that of the others, all estimations of treatment were reduced to the content of the active substance, antimony.

TABLE III.

Kala-Azar Blood Cultures; Untreated Cases.

Specimen No.	Case No.	Spleen or blood smear.	Result of culture.	Proportion of cultures positive.	Globulin test.	Specimen No.	Case No.	Spleen or blood smear.	Result of culture.	Proportion of cultures positive.	Globulin test.
27′	O.P.D. 7336		+	5/9	Positive.	77′	2195	S pleen +	+	3/6	+++
32′	1283		+	4/5	"	80′	O.P.D. 15805		+	3/5	++++
33′	1311		+	2/2	"	83′	" 16059		+	4/6	++++
36′	1390		+	3/4	"	84′	2312		+	1/4	++++
37′	1390		+	6/6	"	85′	2323		+	1/3	++++
48′	1845		+	1/2	"	86′	O.P.D. 16155		+	5/6	+++
49′	1699		−	0/2	"	88′	" 16335		+	3/4	++++
50′	1671	Spleen +	+	1/6	"	91′	S.D.H.		+	1/4	++++
51′	1671	" +	−	0/6	"	92′	2467	Spleen +	+	1/3	++++
53′	1699		+	4/5	"	97′	2669		+	3/4	++
56′	1757		+	3/4	"	98′	O.P.D. 17813		+	2/5	++++
57′	O.P.D. 12149		+	1/4	++++	99′	2572	Spleen +	+	1/3	++++
63′	1881	Spleen +	+	3/3	±	103′	O.P.D. 18404		+	3/4	++++
65′	O.P.D. 14081		+	1/5	+	106′	2751	Spleen +	−	0/3	++++
67′	1995	Spleen +	+	1/5	++	138′	3277	Blood +	+	4/5	++++
70′	O.P.D. 13010		+	2/2	++	166′*	3537	" +	+	1/1	++++
75′	2137	Spleen −	+	4/5	+++	168′*	3537	" +	+	5/6	++++

34 specimens from 30 patients. 31/34 (91.2 per cent) specimens positive; 82/147 (55.8 per cent) cultures positive.

* Cultures made from bottommost blood as well as platelet fraction.

TABLE IV.

Kala-Azar Blood Cultures; Treated Cases.

Treatment up to 12 Mg. of Antimony per Kilo of Patient's Weight.

Specimen No.	Case No.	Amount of antimony received before culture.	Amount of antimony per kilo of patient's weight.	Period covered by treatment.	Interval since last dose.	Weight of patient.	Result of culture.	Proportion of cultures positive.	Order.	Remarks.
		gm.	*mg.*		*days*	*kg.*				
34′	1283	0.007	0.158	(1 dose.)	1	44.3	+	3/3	1	
				hrs.	*hrs.*					
171′	3537	0.00715	0.19	3	3	37.55	+	4/4	2	
					days					
124′	3333 20820	0.0076	0.32	(1 dose.)	3	24	−	0/3	3	Spleen smear: four Leishman-Donovan bodies in four slides.
118′	20503	0.0076	0.49	(1 ")	2	15.5	−	0/5	4	Smear +.
				days						
110′	2282	0.0342	1.26	2	4	27	−	0/5	5	Spleen culture −; 4 cc. of blood.
119′	2984	0.04862	1.43	3	3	34	−	0/2	6	Reported after 20 days.
133′	3333	0.038	1.81	7	3	21	−	0/4	7	The same case as No. 124.
38′	1283	0.1317	2.97	13	3	44.2	+	4/5	8	
41′	1283	0.1317	2.97	13	16	44.2	−	0/2	9	
43′	1283	0.1317	2.97	13	20	44.2	+	2/2	10	
					hrs.					
40′	1390	0.173	5.767	13	8	30 (estimated).	+	1/1	11	
					days					
42′	1390	0.201	6.70	15	2	30 "	+	1/3	12	
102′	1757	0.4719	10.044	49	79	47	+	1/3	13	
39′	118	0.539	10.978	34	3	49	+	1/2	14	
100′	2467	0.3864	11.963	15	1	32.3	+	4/5	15	

15 specimens from 11 patients, each with a total treatment of 0.0158 to 11.963 mg. of antimony per kilo of weight of patient. 9/15 (60 per cent) specimens positive; 21/49 (42.9 per cent) cultures positive.

TABLE V.

Kala-Azar Blood Cultures; Treated Cases.

Treatment above 12 Mg. of Antimony per Kilo of Patient's Weight.

Specimen No.	Case No.	Amount of antimony received before culture.	Amount of antimony per kilo of patient's weight.	Period covered by treatment.	Interval since last dose.	Weight of patient.	Result of culture.	Proportion of cultures positive.	Order.	Remarks.
		gm.	*mg.*	*days*	*days*	*kg.*				
82′	691	0.7541	20.66	95	141	36.5	—	0/4	16	
94′	1881	0.2976	22.718	79	3	13.1	—	0/5	17	
125′	1881	0.3441	22.79	97	80	15.1	—	0/4	18	
132′	3503	1.2616	23.36	123	3	54.0	—	0/4	19	
47′	390	1.108	25.011	222	53	44.3	—	0/2	20	
58′	786	1.52	26.435	149	7	57.5	—	0/3	21	
89′	390	1.1869	26.792	222	109	44.3	—	0/2	22	
74′	691	0.7541	27.622	95	119	27.3	—	0/6	23	
93′	1671	1.2870	28.156	109	3	45.0	—	0/7	24	
108′	2467	0.7704	28.22	47	2	27.3	—	0/5	25	
157′*	3503	1.63513	28.68	151	3	57.0	—	0/4	26	
79′	S. D. H.	0.6851	31.427	131	1	21.8	—	0/10	27	
134′	786	1.61876	34.22	149	206	47.3	—	0/5	28	
117′	1671	1.542	35.69	172	24	43.2	—	0/4	29	
78′	S. D. H.	0.9405	36.313	131	1	25.9	—	0/9	30	
96′	" " "	1.0397	40.143	162	1	25.9	—	0/4	31	
126′	16059	0.65195	40.75	121	6	16.0	—	0/5	32	
95′	S. D. H.	0.9263	42.491	162	1	21.8	—	0/5	33	
123′	2137	1.1583	42.90	131	5	27.0	—	0/2	34	
129′	2137	1.29558	46.77	140	4	27.7	—	0/4	35	
64′	158	0.81	56.643	164	108	14.3	—	0/3	36	
153′*	2137	1.44198	57.68	173	15	25.0	—	0/4	37	
163′*	2195	1.05985	61.62	177	2	17.2	—	0/4	38	
116′	14081	2.21364	67.08	147	2	33.0	—	0/2	39	
156′*	2669	1.39768	77.64	123	3	18.0	—	0/5	40	

25 specimens from 15 patients with a total treatment of 20.66 to 77.64 mg. of antimony per kilo of patient's weight. 0/25 specimens positive; 0/112 cultures positive; *i.e.*, all cultures negative.

* Cultures made from bottommost blood as well as platelet fraction.

tubes contaminated by bacteria were omitted, as flagellates almost invariably disappear from such cultures.

Tables VI to VIII are similar but show the results from cultures from spleen juice obtained by aspiration (spleen puncture). In all these experiments certain specimens were cultured wholly or in part by the addition of the bottommost part of the red cell fraction after centrifugalization to the platelet fraction. The purpose of this will be noted later. They include 108 cultures from 22 specimens from untreated cases and 65 cultures from 12 specimens from treated cases. The net total of spleen cultures was 173 from 34 specimens from 28 patients.

Table IX summarizes the results presented in Tables III to VIII.

A culture method based on the experiments previously noted having been developed, a comparison was made between the results thus obtained and those by Row's technique. The only deviation from his procedure was that the same quantities of blood were taken as with our own method. The experiments were conducted as follows:

10 cc. of blood were drawn into 2 cc. of citrated Locke's solution as before. After dilution in 50 to 70 cc. of the same fluid the contents of the flask were divided into two equal portions. The first was treated as previously indicated (Series A). The second was poured into another similar 50 cc. centrifuge tube and placed in the cool incubator at 22°C. over night (15 to 20 hours). The diluted blood was found to have divided by sedimentation into two layers, similar to those obtained by centrifugalization. There was a cloudy fluid above, and at the bottom a well defined sediment of red cells. The latter was held to represent the material planted by Row. The fluid above corresponded to the fraction planted by us. This was removed by means of a large sterile pipette and set aside. After gentle agitation to cause even distribution of the cells, about 4 drops of the sediment were planted on another series of tubes of medium (Series B). Finally, the cloudy fluid above Row's sediment was centrifugalized and the sediment thus obtained was distributed among a third series of tubes (Series C). Table X shows the results.

The fact that more positive cultures were obtained from Row's sediment than from the platelet fraction above it suggested a study of the different portions of centrifugalized blood diluted with Locke's solution.

TABLE VI.

Kala-Azar Spleen Cultures; Untreated Cases.

Specimen No.	Case No.	Spleen or blood smear.	Result of culture.	Proportion of cultures positive.	Globulin test.	Specimen No.	Case No.	Spleen or blood smear.	Result of culture.	Proportion of cultures positive.	Globulin test.
2	D 3118	Spleen +	+	3/4		33	1311	Spleen +	+	2/6	++
7			+	5/10		50	1671	" +	+	4/4	Positive.
8	288	" +	−	0/6		53	1699	" +	+	5/5	++++
16	573	" −	−	0/6	Positive.	56	1757	" +	+	2/2	Positive.
17	689	" +	+	5/5		63	1881	" +	+	1/3	±
18	691	" +	+	7/7	+	69	1995	" +	+	1/2	Positive.
20	786	" +	+	4/5	Positive.	75	2137	" +	+	2/7	++++
21	744	" −	+	2/6	"	77	2195	" +	+	4/5	+++
23	819	" +	+	6/6	+	92	2467	" +	+	3/5	++++
24	832	" +	+	7/7	Positive.	99	2572	" +	+	1/1	++++
32	1283	" +	+	3/3	+++	106	2571	" +	+	2/3	++++

22 specimens from 22 patients. 20/22 (90.9 per cent) specimens positive; 69/108 (63.9 per cent) cultures positive.

TABLE VII.

Kala-Azar Spleen Cultures; Treated Cases.

Treatment up to 12 Mg. of Antimony per Kilo of Patient's Weight.

Specimen No.	Case No.	Amount of antimony received before culture.	Amount of antimony per kilo of patient's weight.	Period covered by treatment.	Interval since last dose.	Weight of patient.	Result of culture.	Proportion of cultures positive.	Order.	Remarks.
		gm.	*mg.*	*days*	*days*	*kg.*				
1	D 2975	0.026	0.049	3	2	52.7	+	7/14	1	
34	1283	0.007	0.158	(1 dose.)	1	44.3	+	6/6	2	
22	744	0.014	0.77	(1 ")	1	18.0	+	2/3	3	
110	2882	0.0342	1.266	2	4	27.0	−	0/5	4	Blood culture, also "much blood."
122	2984	0.07007	2.06	7	2	34.0	−	2/4	5	
9	157	0.126	5.3	16	63	23.6	+	7/7	6	
5	D 3118	0.24	6.6	24	1	36.3	+	0/8	7	
147	3333	0.02202	9.17	31	12	24.0	+	1/2	8	Four Leishman-Donovan bodies in four slides.
12	157	0.24	10.2	112	1	23.4	+	5/7	9	

9 specimens from 8 patients, each with a total treatment of 0.049 to 10.2 mg. of antimony per kilo of patient's weight 7/9 (77 per cent) specimens positive; 30/56 (53.6 per cent) cultures positive.

TABLE VIII.

Kala-Azar Spleen Cultures; Treated Cases.

Treatment above 12 Mg. of Antimony per Kilo of Patient's Weight.

Specimen No.	Case No.	Amount of antimony received before culture.	Amount of antimony per kilo of patient's weight.	Period covered by treatment.	Interval since last dose.	Weight of patient.	Result of culture.	Proportion of cultures positive.	Order.	Remarks.
		gm.	*mg.*	*days*	*days*	*kg.*				
28	573	0.626	12.9	67	1	48.2	−	0/3	10	
11	D 3134	0.957	30.7	95	2	31.1	−	0/5	11	
155	2137	2.50218	95.87	157	12	26.1	−	0/1	12	

3 specimens from 3 patients, each with a total treatment of 12.9 to 95.87 mg. of antimony per kilo of patient's weight. 0/3 specimens positive; 0/9 cultures positive; *i.e.*, all negative.

TABLE IX.

Kala-Azar.

Summary of Blood and Spleen Cultures.

	Untreated.		Treatment up to 12 mg. per kilo of patient's weight.		Treatment above 12 mg. per kilo of patient's weight.	
	Blood (see Table III).	Spleen (see Table VI.).	Blood (see Table IV).	Spleen (see Table VII).	Blood (see Table V).	Spleen (see Table VIII).
Proportion of specimens positive.....	31/34	20/22	9/15	7/9	0/25	0/3
Percentage " " "	91.2	90.9	60.0	77.0	0.0	0.0
Proportion " cultures "	82/147	69/108	21/49	30/56	0/112	0/9
Percentage " " "	55.8	63.9	42.9	53.6	0.0	0.0

Total cultures, 481 from 108 specimens from 54 patients.

TABLE X.

Comparison of the Centrifuge Method and Row's Sedimentation Method.

Specimen No.	Cultures positive.		
	Series A. Centrifugalized platelet fraction from 5 cc. of blood.	Series B. Row's sediment from 5 cc. of blood.	Series C. Centrifugalized platelet fraction above Row's sediment from 5 cc. of blood.
65′	1/5	0/5	0/5
67′	1/5	0/4	0/4
70′	2/2	3/3	3/3
75′	4/5	3/4	1/2
77′	3/6	0/1	3/4
83′	4/6	3/4	1/3
84′	1/4	1/4	0/2
86′	5/6	1/3	1/3
91′	1/4	1/1	1/2
98′	2/5	0/4	0/4
99′	2/4	0/3	0/1
100′	2/7	2/4	0/4
102′	1/3	2/3	0/3
Total.....	30/69	16/46	10/43
Per cent.....	43.5	34.8	23.3

In blood smears the Leishman-Donovan body is usually found in polymorphonuclear or in large mononuclear leucocytes. Occasionally extracellular forms are encountered. In microscopic examination of stained preparations from the platelet fraction of blood showing positive cultures, extracellular forms were found, though rarely, but intracellular never. Attempts to discover the latter in smears from the buffy coat of leucocytes lying above the red cells after centrifugalization at first disclosed none. Later a few were found. Preparations obtained by removing a small volume of blood from the bottommost part of the tube, however, yielded positive results with five consecutive specimens from three different patients.

A fine capillary pipette, with a square tip and fitted with a small rubber bulb, was plunged directly to the bottom of the centrifuge tube, to avoid drawing in cells from the upper layers. By cautious release of pressure on the bulb, a column of blood 1 to 2 cm. high was removed, and smears were made on slides. Care was taken to use all of the drop of blood before the farther end of the slide was reached; otherwise many leucocytes were crowded into the thick windrow of cells at that place, and an examination of these was impossible. The ideal smear is one that does not quite reach the edges of the slide and has an unbroken rounded end. The smearing was done with another slide in the usual manner. Wright's or Leishman's stain was used as for ordinary blood preparations; *i.e.*, the undiluted stain was allowed to act for 1 minute to fix the film, an equal volume of distilled water was then added, and after 4 minutes the diluted stain was floated off, with distilled water, care being taken that no sediment settled on the film. The slide was washed for about 30 seconds and then blotted with smooth filter paper. It was examined by means of an oil immersion lens and a Bausch and Lomb No. 5 eyepiece. This was exchanged for a Bausch and Lomb No. 10 or a Leitz No. 12 ocular when a doubtful object was encountered. By means of a mechanical stage the whole of each slide was carefully searched. As the time consumed was from 1 to 5 hours per slide, not all smears from each case were searched. While centrifugalization is the most convenient method of obtaining the sediment, smears obtained from the bottom of tubes of diluted blood allowed to stand for 7 to 12 hours have given positive results. Pointed centrifuge tubes were used for the purpose, but any similar vessel, such as a urine glass, may be substituted. This reduces the method to very simple terms.

This empirical method, then, was successful, but it remained to be demonstrated whether other portions of the centrifugalized blood were as rich or richer in the leucocytes known to phagocytose the

Leishman-Donovan bodies; *i.e.*, the polymorphonuclear neutrophils and large mononuclear leucocytes. In order to study this question the following experiment was carried out.

10 cc. of blood were drawn from a vein and diluted, as previously described for blood culture, and centrifugalized slowly for 5 minutes. The patient selected (Case 3719) was a convalescent from amebic dysentery and infestation with several other intestinal parasites. His blood count was essentially normal. 2 days previous to the experiment it had shown: leucocytes 8,200; red cells 4,800,000; hemoglobin 100 per cent. Differential count: polymorphonuclear neutrophils 67.5 per cent; polymorphonuclear eosinophils 0.5 per cent; polymorphonuclear basophils 0.0 per cent; lymphocytes 37 per cent; large mononuclears 4 per cent.

TABLE XI.

Differential Counts of Leucocytes in the Different Strata of Centrifugalized Diluted Blood.

Cells.	Platelet fraction. Two complete smears.	Buffy coat. 400 cells.	Red cell fraction. 400 cells.	Bottommost blood. 400 cells.	Whole blood. 400 cells.
		per cent	*per cent*	*per cent*	*per cent*
Polymorphonuclear neutrophils	18 cells.	67.0	85.0	91.0	59.6
Polymorphonuclear eosinophils	0	1.0	2.0	4.0	2.3
Polymorphonuclear basophils	0	0.8	0.0	0.0	0.2
Lymphocytes	1 cell.	23.2	11.5	3.0	27.4
Large mononuclears	1 "	8.0	1.0	2.0	10.5
Platelets	Very many.	Few.	Very few.	Almost none.	Normal.
Leucocytes	Almost none.	Many.	Few.	Numerous.	"

1. After the first slow centrifugalization the supernatant cloudy fraction was pipetted off to another pointed 50 cc. centrifuge tube and spun rapidly for 10 minutes. Cover-glass smears were made from the sediment obtained. These were taken to represent the platelet fraction.

2. The first tube showed on top of the red cells a yellowish film (buffy coat). This was suspended in a few cubic centimeters of Locke's solution, added by means of a pipette without disturbing the sediment. Very gently agitation was accomplished by pressing the pipette bulb. The cloudy fluid was removed to a pointed 15 cc. centrifuge tube, spun rapidly, and cover-glass smears were made from the sediment. These were called the buffy coat.

3. A capillary pipette was now introduced into the center of the red cell sediment remaining in the first tube and a column of corpuscles about 2 cm. long was

withdrawn and smears were made on cover-glasses. They were taken to represent the red cell fraction.

4. Finally, a capillary pipette was run to the bottom of the original tube and a 2 cm. column of sediment removed. Cover-glass smears were made as before. These were called bottommost blood.

5. At the same time smears were made from the whole blood (ear puncture) for a control differential count.

TABLE XII.

Leishman-Donovan Bodies in the Buffy Coat and the Bottommost Blood.

Specimen No.	Smear.	No. of slides examined.	Total No. of cells containing Leishman-Donovan bodies.	Total No. of Leishman-Donovan bodies contained.	Average No. of cells containing Leishman-Donovan bodies per slide.	Average No. of Leishman-Donovan bodies per slide.	Remarks.
142′	bc*	4	1	1	0.25	0.25	
	bb†	4	8	12	2.00	3.00	
168′	bc	4	1	7	0.25	1.75	Before adrenalin, 0.5 cc.
	bb	4	21	22	5.25	5.50	
171′	bc	4	6	30	1.5	7.5	1 large mononuclear containing 7 Leishman-Donovan bodies; 1 large mononuclear containing 16 Leishman-Donovan bodies. Patient received 5 cc. of 0.2 per cent Sb_2S_3 2 hrs. before receiving adrenalin, 0.5 cc. Specimen taken 30 min. after injection of adrenalin.
	bb	4	8	10	2.0	2.5	
175′	bc	4	1	1	0.25	0.25	Before adrenalin, 0.5 cc.
	bb	4	4	4	1.00	1.00	
176′	bc	4	2	8	0.50	2.00	30 min. after adrenalin, 0.5 cc.
	bb	4	10	18	2.50	4.50	
Total...	bc	20	11	47	0.55	2.35	
	bb	20	51	66	2.55	3.3	

Proportion of infected cells in buffy coat to infected cells in bottommost blood, 1/4.6.

* bc, buffy coat.

† bb, bottommost part of red cell fraction; *i.e.*, bottom of tube.

All the smears were stained by Leishman's method and counted by means of a mechanical stage. Except for the platelet fraction, the differential count was based on 400 cells. Table XI shows the results.

The leucocytes known to phagocytose Leishman-Donovan bodies were mostly in the buffy coat and at the bottom of the tube. They were also in the red cell fraction, but were few compared with the number of red cells in the same smear or with the absolute numbers of leucocytes in slides from the other two portions. The buffy coat

TABLE XIII.

Leishman-Donovan Bodies in the Platelet Fraction.

Specimen No.	Smear.	No. of slides examined.	Total No. of Leishman-Donovan bodies found.	Average No. of Leishman-Donovan bodies per slide.	Remarks.
142′	Platelet fraction.	4	0	0.0	
175′	Platelet fraction	4	2	0.5	Before adrenalin, 0.5 cc.
176′	Platelet fraction.	4	0	0.0	30 min. after adrenalin, 0.5 cc.
Total...		12	2	0.17	

No intracellular forms and very few leucocytes found.

and the bottommost fraction therefore seemed the most hopeful places to search for infected cells. Smears were made from the buffy coat (bc) and from the bottommost part of the tube (bb) from cases which had shown intracellular parasites in the latter fraction. Four slides of five specimens from three different patients were examined from these two portions. The results are shown in Table XII.

The average number of infected cells per slide was taken to be an index of the value of the two parts of the blood for diagnosis by

TABLE XIV.

Blood Smears; Bottommost Blood.

Specimen No.	No. of slides examined.	No. of slides positive.	Treatment to date of examination. Amount of antimony per kilo of patient's weight.	Result of culture.	Proportion of cultures positive.	Remarks.
			mg.			
137′	3	3	0.00	Contaminated.		Patient admitted for dysentery (proved *B. dysenteriæ* Shiga). Globulin ± or +. The same case as Nos. 142′, 143′, and 151′.
138′	3	3	0.00	+	4/5	Patient died 12 hrs. later; cancrum oris. 25 polymorphonuclear leucocytes in 1 slide contained Leishman-Donovan bodies.
166′	3	1	0.00	+	1/1	Mother and brother had kala-azar. The same case as Nos. 168,′ 171,′ 177′, and 178′.
168′	2	2	0.00	+	5/6	Mar. 11, 1922, 10 a.m. 1 slide: 11 polymorphonuclear leucocytes contained 1 Leishman-Donovan body each. 1 slide: 3 polymorphonuclears contained 1 Leishman-Donovan body each. The same case as No. 171′.
171′	4	4	0.00	+	4/4	11.45 a.m. 5 cc. of 0.2 per cent colloidal Sb_2S_3. 1.50 p.m. 0.5 cc. of adrenalin solution. 2.20 p.m. Blood specimen taken 4 slides: 3, 1, and 1 Leishman-Donovan bodies in 3, 1, and 1 polymorphonuclears; 11 Leishman-Donovan bodies in 1 large mononuclear.
173′	1	1	0.00			1 slide: 18 Leishman-Donovan bodies in 16 polymorphonuclears.
174′	6	2	0.00			2 slides: 1 Leishman-Donovan body in polymorphonuclear in each. The same case as Nos. 175′ and 176′.

175′	2	2	0.00			2 slides: 2 and 1 Leishman-Donovan bodies in 2 and 1 polymorphonuclears. Immediately before adrenalin, 0.5 cc. The same case as Nos. 176′ and 174′.
176′	3	3	0.00			3 slides: 5, 3, and 10 Leishman-Donovan in 3, 2, and 4 polymorphonuclears. 30 min. after adrenalin. The same case as Nos. 175′ and 174′.
118′	3	2	0.49	—		2 slides: 3 and 11 Leishman-Donovan bodies in 1 and 1 large mononuclear leucocytes.
142′	7	7	0.55	Not made.		The same case as Nos. 137′, 143′, and 151′.
143′	4	3	1.10	" "		" " " " " 137′, 142′, " 151′.
177′	4	0	1.71			" " " " " 166′, 168′, 171′, and 178′. Immediately before adrenalin, 0.5 cc.
178′	4	1	1.71			1 slide: a Leishman-Donovan body in 1 polymorphonuclear leucocyte 30 min. after adrenalin, 0.5 cc.
151′	2	2	4.34	Contaminated.		The same case as Nos. 137′, 142′, and 143′. 4 blood specimens; all positive. 2 cultures both contaminated.
145′	2	0	5.13	"		2 previous blood cultures negative. 4 smears from spleen puncture showed 3 and 1 Leishman-Donovan bodies in 1 and 1 large mononuclear leucocytes.
157′	2	0	28.68	—		
149′	2	0	52.97	Contaminated.		Spleen and blood cultures positive before treatment. The same case as Nos. 75′, 75, 123′, 153′, and 155.
153′	2	0	57.68	—		The same case as No. 149′.
163′	2	0	61.62	—		Spleen and blood cultures positive before treatment. The same case as Nos. 77′ and 77.
148′	2	0	71.80			Blood culture positive before treatment. The same case as Nos. 65′, 116′, and 130′.
156′	2	0	77.64	—		Blood culture positive before treatment. The same case as Nos. 97′ and 158′.
158′	2	0	77.64	Contaminated.		The same case as No. 156′.

stained blood smears. It is seen from the table that in the present series the bottommost blood slides contained 4.6 times as many infected leucocytes as the buffy coat.

From three of the blood specimens used in preparing the smears for the counts shown in Table XII slides were also made from the centrifugalized platelet fraction. Four smears from each specimen were examined for Leishman-Donovan bodies. The result is shown in Table XIII.

Since this method of examination of blood from kala-azar patients was devised it has been used with twenty-three specimens from twelve different cases. In Table XIV these are arranged according

TABLE XV.

Summary of Blood Smears; Bottommost Blood.

Specimens.	No. of specimens examined.	No. of slides examined.	No. of slides positive.	Percentage of specimens positive.	Percentage of slides positive.
Untreated cases	9	27	21	100.0	77.8
Treated with less than 12 mg. of antimony.*	7	26	15	71.4	57.7
" " over 12 mg. of antimony	7	14	0	0.0	0.0

* It will be noted from Table XIV that in this comparatively small series no parasites were found in smears from patients who had received more than 4.34 mg. of antimony per kilo of body weight.

to treatment, in ascending order of milligrams of antimony per kilo of patient's weight.

The results are summarized in Table XV, in which the specimens are divided on the basis of results from cultures, as shown in Tables III to IX; *i.e.*, (*a*) untreated, (*b*) treated up to 12 mg. per kilo of patient's weight, and (*c*) treated with more than 12 mg. per kilo.

DISCUSSION.

The reason for inconstant or negative results with media containing human blood becomes evident from a study of Tables I and II. No growth of *Leishmania* occurred when a salt agar medium containing human serum was used. The same effect is shown in Table

X. The only essential difference between Series A and C is that in the latter the organism in the platelet fraction, *i.e.* extracellular forms, were in contact with the patient's serum diluted with citrated Locke's solution for 15 to 20 hours. The number of positive cultures as a result of this was reduced nearly half. From Tables I and II it is seen that there was little or no growth when washed human red cells were substituted for rabbit blood, but the effect was not so marked as when human serum was used. Horse and sheep serum and cells occupy a position between the blood of man and that of the rabbit. Media containing either rabbit cells or serum gave positive results, though not to the extent of the whole defibrinated blood of the control tubes. Heating to 56°C. for 1½ hours had no appreciable effect.

The blood culture method devised was based on these data. It seemed desirable to use a larger volume of blood for culture than those previously employed, provided the portion containing the organism could be separated from the serum and red cells. The technique already described proved satisfactory. The results are shown in Tables III, VI, and IX. In untreated cases of kala-azar it has been possible to obtain positive cultures in one or more tubes in 91.2 per cent of the thirty-four specimens taken. When compared with the spleen juice series it is seen that the blood gave a slightly better showing. The number of tubes planted which proved positive shows a slight advantage for the spleen cultures—63.9 against 55.8 per cent. It should be noted, however, that most of these cultures were made from the platelet fraction only, which contains no intracellular parasites. In a word, by this technique blood cultures are as dependable as those from spleen puncture, provided a sufficient number of culture tubes is used.

After the finding of intracellular forms in the bottommost portion of the red cell fraction this material was added to the platelet fraction after the first centrifugalization. The resultant combined sediment was planted. Two series of cultures have been begun, one with the previous technique and the other with the technique just outlined. The number of cultures studied thus far by the two methods is not large enough to show their relative values.

The inconstancy of previous methods seems to lie in the small volume of blood taken and in the inhibitory action of the human

blood constituents necessarily added to the culture. Mayer and Werner (12) used whole blood, while Row added only the red cell fraction obtained by sedimentation. The latter, in attempting to avoid the deleterious effect of the serum by diluting it, probably killed some of the organisms by prolonged contact during the period of sedimentation. The present experiments do not shed any light on the question of whether there was any reduction in intracellular forms in the sediment. They do show that the extracellular forms in the fluid above did not grow so frequently after 15 to 20 hour sedimentation as did those from the similar material obtained by immediate centrifugalization after dilution.

The effect of treatment with antimony on the cultivability of *Leishmania* from the blood remains to be considered. Tables IV and V really form a continuous series of treated cases. The cases were divided, as previously explained, at the point where positive cultures ceased. The spleen culture series was similarly dealt with, and the division point coincides closely. The specimens yielding negative cultures in Table IV deserve brief notice. Specimens 124′ and 133′ were from a case in which the spleen puncture culture (Table VII, No. 147) and smears were positive. The latter, however, showed only four Leishman-Donovan bodies in four slides, so that the infection must have been light. Specimen 118′ has been noted as one in which the blood smear was positive. Only the platelet fraction was planted. Specimen 110′ consisted of only 4 cc. of blood. The spleen culture (Table VII, No. 110) taken at the same time was also negative. With Specimen 119′ only two tubes remained uncontaminated. Both were negative. The report was made after 20 days incubation. Occasionally cultures became positive later than this.

Table V shows a series of cultures from blood specimens of patients who had received from 20.66 to 77.64 mg. of antimony per kilo of body weight. The blood was withdrawn from 1 to 206 days after the last dose. None of the cultures was positive, although the interval was over 100 days in five cases. It is not contended that a total treatment of 12 mg. of antimony per kilo of the patient's weight is a sharp line beyond which positive cultures cannot be obtained. Neither is it to be maintained that such a dose is curative

in all instances. That the patient has become sterilized after approximately this dose is certainly suggested, though a much larger series is needed to prove the point. That antimony is not immediately bactericidal is shown in two cultures. Specimen 40′ (Table IV), taken 8 hours after the patient had received 20 cc. of a 0.2 per cent suspension of colloidal antimony sulfide, gave a positive culture. Specimen 171′ was taken 2½ hours after 5 cc. of the same preparation had been received. All four culture tubes were positive.

Even though a culture method be developed that shows positive results in 90 per cent of untreated cases, the delay of 1 to 3 weeks before the flagellates appear makes a more rapid method of diagnosis desirable. Blood smears have been positive in the hands of many workers, but the number of slides examined and the time required, if the examination is carefully done, makes this method impracticable. The finding of intracellular forms in the bottommost part of the red cell fraction appears to offer a better chance of detecting the Leishman-Donovan body than does a smear made directly from a drop of peripheral blood. The technique suggested concentrates the leucocytes containing the parasites, so that most of them from 5 cc. of blood are collected onto a few slides.[8] From the smears thus far examined from untreated cases it is evident that the number of organisms present varies between wide limits. Blood Specimen 138′ was taken from a child with cancrum oris who died within a few hours. In some smears from the bottom of the red cell fraction polymorphonuclears containing Leishman-Donovan bodies were found in almost every field. In one such preparation, three adjacent cells contained one, two, and three parasites respectively. On the other hand, in Specimen 166′ three slides were examined. The first showed one Leishman-Donovan body in one polymorphonuclear, but the next two smears were negative. No. 118′ is worthy of note, because while a mononuclear containing parasites was found in each of two slides out of three examined, the

[8] It may be noted by way of parenthesis that the presence of Leishman-Donovan bodies in the endothelial cells *in situ* on the vessel walls (Christophers (4)) and in similar cells in the blood stream offers evidence of the endothelial origin of at least some of the large mononuclear cells of the blood.

culture was negative. As only the platelet fraction was planted, the negative culture probably means that there were no extracellular organisms in the material planted. Nos. 137′ and 151′ are of interest for two reasons. The patient, a child, was admitted for dysentery, which was proved to be due to the Shiga bacillus. During the physical examination the spleen was found to be just palpable. The globulin precipitation test was faintly positive. Stained smears made from the bottom of the red cell fraction at the time of culture showed Leishman-Donovan bodies in polymorphonuclear leucocytes after about 15 minutes search. The positive diagnosis of kala-azar resulted in immediate intravenous treatment. This gave the patient a better chance of recovery from the secondary infection of dysentery as well as from the primary disease. Secondly, both blood cultures from this patient were contaminated, so that had culture alone been depended upon for diagnosis no information would have been obtained. The blood smears from blood specimens taken on 4 different days were all positive.

From our experience thus far, blood smears appear to be a valuable means of diagnosis. When the organisms are present in the blood in moderate or large numbers a rapid diagnosis may be made. If they are few, the time consumed in searching is too great. It is then better to rely on the globulin precipitation test (20, 21) for presumptive evidence, and if the result be positive, to begin treatment while awaiting the result of the blood culture. In the Peking Union Medical College Hospital the following is the present procedure. Two series of cultures are made from each 10 cc. blood specimen. The first is from the platelet fraction only. The second is from this together with the bottommost part of the red cell fraction. This leaves the red cell fraction from one tube intact. Smears are made on slides from the bottom of this tube, stained, and examined. In this way one 10 cc. specimen of blood is utilized for making one series of blood smears and two series of blood cultures. The combined results offer the maximum of information. In addition, if desired, a globulin precipitation test may be made from the citrated blood remaining in the syringe after expulsion of the specimen into the flask of Locke's solution.

SUMMARY.

1. The effect of human red cells and serum on the growth of *Leishmania donovani* has been studied and a blood culture method devised, based on these findings.

2. The distribution of blood platelets and of the different varieties of leucocytes in the strata of centrifugalized diluted blood has been investigated, and also the distribution of Leishman-Donovan bodies, extracellular and intracellular, in these strata.

3. The effect of treatment with antimony on the cultivability of *Leishmania donovani* has been studied.

CONCLUSIONS.

1. N. N. N. media in which human red cells replace the rabbit blood show only a feeble growth of *Leishmania donovani.*

2. No growth occurs on salt agar to which human serum, whether inactivated or not, has been added.

3. If an adequate amount of blood is taken (10 cc.) and most of the serum and red cells removed by centrifugalization, positive cultures may be obtained in about 90 per cent of untreated cases of kala-azar.

4. By this technique positive cultures may be obtained from the peripheral blood in at least as many cases as from spleen punctures.

5. Extracellular Leishman-Donovan bodies are found in the platelet fraction of centrifugalized diluted blood.

6. Mononuclear and polymorphonuclear leucocytes, with the contained intracellular forms, are usually heavier than the other blood cells and go to the bottom of the tube on centrifugalization or sedimentation. Leishman-Donovan bodies may be recovered from this fraction.

7. In the series of cultures made, the blood of patients treated intravenously with antimony salts became free from cultivable *Leishmania donovani* after they had received about 12 mg. of antimony per kilo of body weight.

8. Cultures from spleen punctures show similar results.

BIBLIOGRAPHY.

1. Donovan, C., *Lancet*, 1904, ii, 744.
2. Laveran, A., and Mesnil, F., *Compt. rend. Acad.*, 1904, cxxxviii, 187.
3. Laveran, A., and Cathoire, *Bull. Acad. méd.*, 1904, li, 247.
4. Christophers, S. R., *Sc. Mem. Off. Med. and San. Dept. Gov. India*, 1904, N. S., No. 11.
5. Knowles, R., *Indian J. Med. Research*, 1920–21, viii, 162.
6. Rogers, L., *Proc. Roy. Soc. London, Series B*, 1906, lxxvii, 284.
7. Cornwall, J. W., and LaFrenais, H. M., *Indian J. Med. Research*, 1915–16, iii, 698.
8. Wylie, J. H., *China Med. J.*, 1920, xxxiv, 593.
9. Knowles, R., *Indian J. Med. Research*, 1920–21, viii, 177.
10. Rogers, L., Fevers in the tropics, London, 3rd edition, 1919, 48.
11. Bramachari, A. N., Kala-azar and its treatment, Calcutta, 2nd edition, 1920, 99.
12. Mayer, M., and Werner, H., *Deutsch. med. Woch.*, 1914, xl, 67.
13. Wenyon, C. M., *J. Trop. Med.*, 1914, xvii, 49.
14. *Indian J. Med. Research*, 1914–15, ii, p. ix.
15. Korke, V., see reference 7, editorial foot-note.
16. Patton, W. S., *Indian J. Med. Research*, 1914–15, ii, 492.
17. Marshall, W. E., *Rep. Wellcome Trop. Research Lab.*, 1911, iv, 157.
18. Cannata, S., quoted by Wenyon, *Trop. Dis. Bull.*, 1922, xix, 1.
19. Mazzoni, L., *Pediatria*, 1921, xxix, 347.
20. Sia, R. H. P., *China Med. J.*, 1921, xxxv, 397.
21. Sia, R. H. P., and Wu, H., *China Med. J.*, 1921, xxxv, 527.

MESENCHYME AND MESOTHELIUM.

By WARREN H. LEWIS, M.D.

(From the Department of Embryology of Carnegie Institution of Washington, Johns Hopkins Medical School, Baltimore.)

PLATES 17 AND 18.

(Received for publication, April 26, 1923.)

During the early embryonic period there is a continual differentiation of the mesenchymal cells into vascular endothelium (Sabin,[1] 1920) and into mesothelium of the various serous cavities. The question is still open "as to the time, if ever, when angioblasts cease entirely to differentiate and all of the growth comes to be from the walls of previously formed vessels, as was observed by E. R. Clark[2] (1909) for the tadpole's tail" (Sabin[3]). The experiments of W. C. Clarke[4] indicate that mesothelium can be formed from the mesenchyme, even in adult life. It seems probable that endothelium is a real differentiation from the primitive mesoderm, while mesothelium is more in the nature of a transformation in the form of the cells, due to environment. If such be the case, one would expect the process of differentiation of primitive mesoderm into endothelium to cease at some time during development, unless the mesoderm, or certain regions of it, remain in this primitive state. The view that mesothelium arises as a transformation in form rather than as a differentiation of the mesenchyme is indicated by the following observations on cultures of the heart mesenchyme.

[1] Sabin, F. R., Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation, *Carnegie Institution of Washington, Pub. No. 272, Contributions to Embryology*, 1920, ix, 213.

[2] Clark, E. R., Observations on living growing lymphatics in the tail of the frog larva, *Anat. Rec.*, 1909, iii, 183.

[3] Sabin,[1] p. 256.

[4] Clarke, W. C., Experimental mesothelium, *Anat. Rec.*, 1915–16, x, 301.

Cultures from the hearts of chick embryos (4 to 11 days incubation) in the Locke-bouillon-dextrose medium[5] often show mesothelial-like membranes of varying extent. Sometimes these membranes migrate from the edge of the explant and remain quite sharply distinct from the mesenchymal reticulum. Such mesothelial membranes are usually large and may constitute the major portion of the outgrowth, extending from the explant (Fig. 1). In some cultures only small patches of mesothelium are encountered (Figs. 2 and 3), and often their borders are not sharply delimited from the surrounding migratory reticular mesenchymal cells (Fig. 3). When the borders of some of these membranes are examined carefully, one can see in both the fixed and living cells all transitions between the ordinary multipolar reticular mesenchymal cell and the flat mesothelial-like cell, which abuts upon its neighbors in fairly sharp lines (Fig. 3) that give the characteristic silver nitrate reaction.

The reverse process, changing from mesothelial to multipolar form, also takes place, especially at the outer edge of the membranes, where cells may become entirely detached. During the early stages of degeneration all the cells of a mesothelial membrane may change to the reticular form and eventually round up more or less and become entirely isolated from one another.

It seems evident that the mesothelial-like cells and the multipolar cells are merely different forms of the same cell type. Conclusive proof of this was obtained by watching the actual transformation of multipolar cells into mesothelial-like cells. It was at the periphery of a small patch of mesothelium, such as is shown in Fig. 3, that this change was observed in a number of cells during the course of 2 or 3 hours. All the cells were more or less flattened against and adherent to the cover-glass. The multipolar mesenchyme cells had varying numbers of processes which overlapped and were adherent to those of neighboring cells. (Some of the reasons for believing that mesenchyme forms an adherent reticulum and not a syncytium have already been indicated in a previous paper.[6]) As the cells

[5] Locke's solution (NaCl 0.9 gm.; $CaCl_2$ 0.024 gm.; KCl 0.042 gm.; $NaHCO_3$ 0.02 gm.; and distilled water 100 cc.) 80 cc., chicken bouillon 20 cc., and dextrose 1 gm.

[6] Lewis, W. H., Is mesenchyme a syncytium? *Anat. Rec.*, 1922, xxiii, 177.

changed from the reticular to the plate-like mesothelial form, the longer processes were partially retracted, while at the same time the body of the cells spread out to meet the edges of neighboring cells. This identity of the multipolar and mesothelial forms is seen at the periphery of the membranes, where one or more edges of the cell may be rather straight and sharp, while the outer side presents the reticular multipolar character (Figs. 3 and 4). Again, in places where the intercellular lines are sharp and clear, the entire thickness of the edge may abut upon the edges of neighboring cells, as at *a* in Fig. 5, or the deeper part of this edge may overlap, so that part of the edge next to the cover-glass may abut sharply, while deeper focusing in the same region shows overlapping (Figs. 4 and 5, *b*). All degrees between sharp abutment of the entire edge and almost complete overlapping of irregular edges of flat cells are to be found in different membranes, and sometimes in different parts of the same membrane. Some show scarcely any intercellular boundaries at all.

The general cytological character of the reticular and the mesothelial cells is similar, as can be seen from the figures. Such differences as exist are those of form rather than structure. The nuclei tend to become more spherical in the mesothelial and more oval in the reticular cells, especially when the latter are elongated. The nuclei are plastic bodies and conform more or less to the shape of the cell. The cytoplasm in the multipolar reticular cells is usually thicker in the region of the nucleus than in the same region of the more spread out mesothelial cells and hence stains more deeply, often obscuring the mitochondria.

The mitochondria are similar in the two forms and show the same variations in size, shape, and distribution. They are few and scattered in the peripheral regions of the cell, numerous and closer together near the nucleus. They vary in shape from threads and rods to granules. In the younger cultures most of the mitochondria are thread-like in the mesothelium and mesenchyme, while in the older cultures they tend to be granular in shape.

The degeneration changes which occur in the older cultures seem to be identical in the two forms. There is a gradual accumulation of granules and vacuoles about the region of the centriole and the

enlarged centrosphere that develops about the centriole. The mitochondria tend to become arranged about the centriole and centrosphere, at first more or less radially; later, if the centrosphere becomes much enlarged, they often change to a concentric position around it.

It seems probable that some of the mesothelial membranes, especially those that migrate out from the explant as such, may come directly from the mesothelium of the epicardium. This source obviously does not account, however, for all the membranes, unless the mesothelium changes to reticulum as it migrates off from the explant and then changes back again to mesothelium. But mesothelium, and the mesenchyme as well, are too common and too abundant for this to be the only source. The reticular cells resemble those encountered in cultures of subcutaneous tissue, but the general reticular outgrowths from the heart are rarely as thick as those from subcutaneous tissue. On this account one can usually distinguish the two at a glance. This difference probably depends upon the fact that heart explants contain fewer mesenchyme cells. On the other hand, I have never seen mesothelial membranes in cultures of subcutaneous tissue. If heart and subcutaneous mesenchyme are identical, why should this be so? The media and cover-glasses are similar, yet the explants differ, causing one to wonder whether there can be substances which diffuse out from the heart that can account for this. Of course, it may be that heart mesenchyme and subcutaneous mesenchyme are really different. In cultures of the intestine mesothelial membranes are occasionally encountered, but they are rarely as well developed and never so extensive as those in heart cultures. Explants of the intestine, however, almost always have on their surfaces much larger areas of mesothelium than heart explants, many of which show only small areas or none at all. This would seem to indicate that the presence of mesothelium on the surface of the explant does not insure its occurrence in the outgrowth.

The possibility of an endothelial origin for much of the reticular mesenchyme in heart cultures should be considered. However, in cultures of the liver (Lewis,[7] 1922), in which endothelium is easy to recognize, since there are practically no mesenchyme cells in the

[7] Lewis, W. H., Endothelium in tissue cultures, *Am. J. Anat.*, 1922, xxx, 39.

embryonic liver at certain stages, no mesothelium was observed. This would imply that the mesothelium in the heart cultures did not come from endothelium. In cultures of other embryonic tissues we have no criteria at present which might enable us to differentiate easily between endothelial and mesenchymal cells in the outgrowth. This may mean either that endothelium reverts to the more primitive form, or that, under the conditions of the cultures, it resembles so closely the mesenchyme in form that it is impossible to distinguish one from the other. One often sees, in cultures of various organs, cells that are probably endothelial, but it is not possible to determine this definitely. The cells that change into mesothelium in heart cultures are certainly mesenchymal in appearance, and sometimes all the cells that migrate out are of this type. They show great variation in form, from long, slender, bipolar cells to multipolar, flat, spread out ones that approach the mesothelial form.

SUMMARY.

The migratory mesenchymal cells in cultures of the embryonic chick heart show all stages of transformation from the bipolar and multipolar reticular cells to the flat mesothelial forms. One can actually observe this change in form. Such mesothelial cells seem to differ from the mesenchymal cells only in form and not in structure, indicating that mesothelium is to be considered not as a tissue differentiated from the mesenchyme, but merely as a change or transformation in form.

These observations, together with those of W. C. Clarke,[4] indicate that, in the healing of wounds of the mesothelium lining the peritoneal, pleural, and serous cavities, new mesothelium may arise from the subjacent mesenchyme by the change in form of its cells, and that the repair after abrasions of the mesothelium is not necessarily brought about by a spreading of the adjacent mesothelium over the wound, such as occurs in repair of skin wounds.

EXPLANATION OF PLATES.

PLATE 17.

FIG. 1. Mesenchyme and mesothelium; 8 day culture; heart of 5 day chick embryo; Locke-bouillon-dextrose medium; Janus green, iodine vapor. × 146.

FIG. 2. Mesenchyme and small patch of mesothelium; 8 day culture; heart of 5 day chick embryo; Locke-bouillon-dextrose medium; Janus green, iodine vapor. × 146.

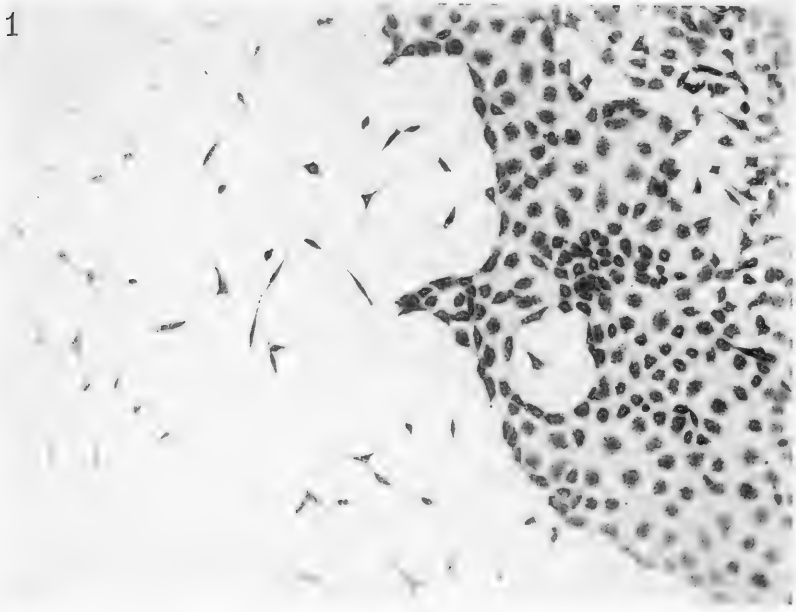
PLATE 18.

FIG. 3. A few mesothelial cells surrounded by transitional mesenchyme; 5 day culture; heart of 6 day chick embryo; Locke-bouillon-dextrose medium; Janus green, iodine vapor. × 480.

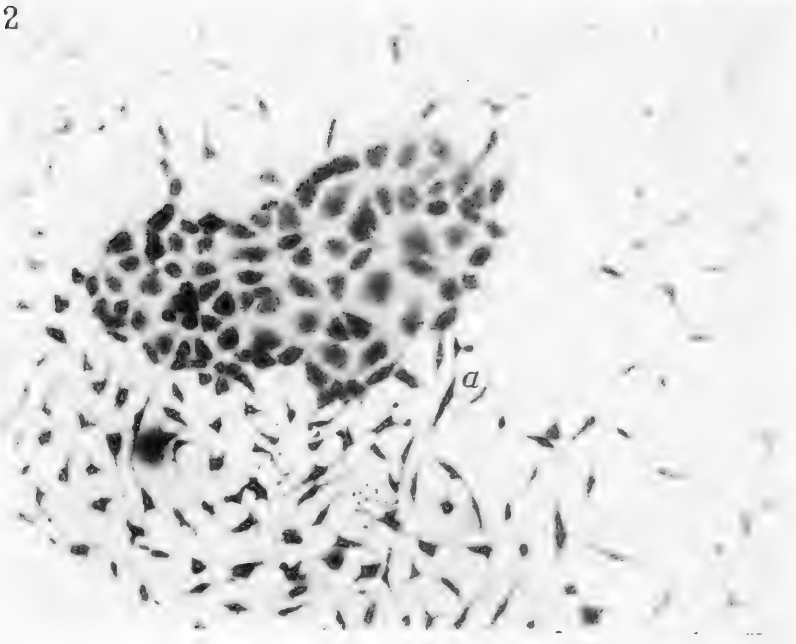
FIG. 4. Edge of mesothelial membrane at *a* in Fig. 2, showing transition cell. × 480.

FIG. 5. Diagrammatic cross-section of part of a cover-glass and mesothelial membrane.

1

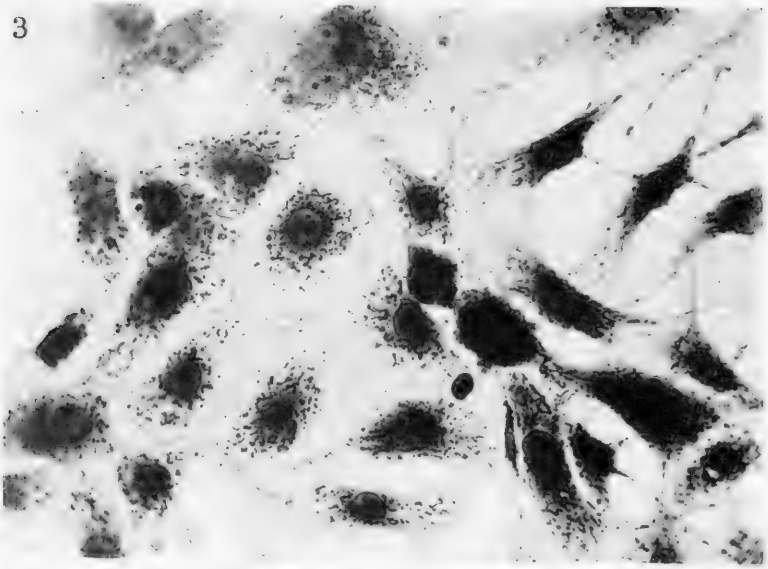


2

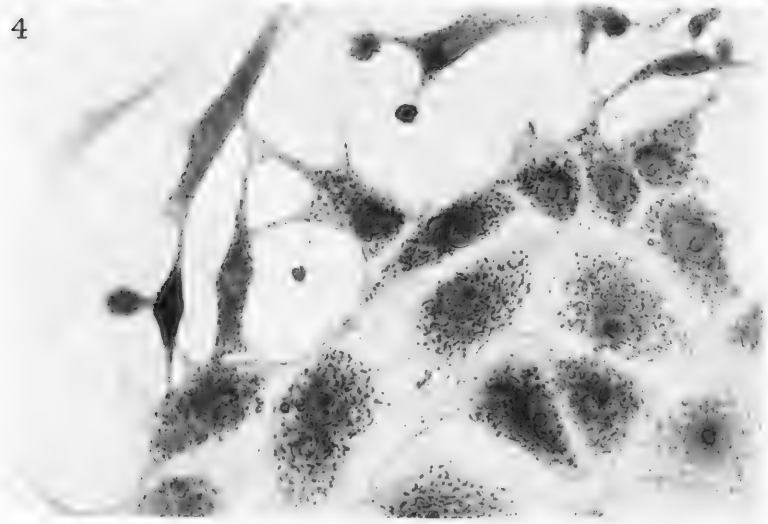


(Lewis: Mesenchyme and mesothelium.)

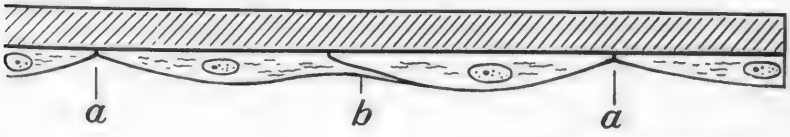
3



4



5



(Lewis: Mesenchyme and mesothelium.)

STUDIES ON ENDOTHELIAL REACTIONS.

VIII. Changes in the Distribution of Tubercle Bacilli and Tubercles in the Organs of Rabbits Following Splenectomy.

By NATHAN CHANDLER FOOT, M.D.

(From the Department of Pathology of the University of Cincinnati College of Medicine and Cincinnati General Hospital, Cincinnati.)

Plates 19 and 20.

(Received for publication, May 5, 1923.)

INTRODUCTION.

It has been found that if a rabbit be splenectomized and injected intravenously with colloidal carbon a week or so later, the greater part of this carbon will be taken up by the pulmonary endothelium, which shows not only an increased ability for phagocytosis, but also proliferative activity. The details of such an experiment were described in a preceding paper of this series (Foot, 1923). This change in the reaction of the pulmonary endothelium to foreign material, as compared with that in control animals, has comparatively little practical significance, as such large quantities of inert matter seldom if ever find their way into the circulation under conditions other than experimental. It would be of greater practical importance to know what effect splenectomy would have on the distribution of microorganisms subsequently introduced into the blood stream.

The tubercle bacillus calls forth a marked endothelial reaction and is frequently found in the circulation under conditions arising spontaneously after the rupture of a tubercle into a vessel. What effect would splenectomy have upon the distribution of this bacterium in experimental general miliary tuberculosis? Would the lesions produced differ from those observed in control rabbits?

EXPERIMENTAL.

In order to answer these questions the following experiment was undertaken.[1] It was found that definite differences in the distribution of the organisms and the character of the resulting lesions do exist, as compared with the findings in normal controls. These differences are sufficiently striking to warrant description and discussion.

The rabbits used were divided into two groups; five were splenectomized (Group S) and five reserved for controls (Group C). They were weighed and injected intravenously, about 2 weeks after the operation, with 0.2 mg. per kilo of a 2 week culture of bovine tubercle bacilli on glycerol agar slants. The suspension was prepared in the usual manner, by rubbing up a weighed amount of tubercle bacilli with salt solution and gradually adding more of the latter until an even suspension was obtained. This was diluted until 1 cc. equalled 0.2 mg. Necropsies were performed upon the animals as soon as they were found dead, the first (Rabbit S1) dying 19 days, the last 1 month after injection. The order of the deaths in the series was as follows: No. S1, 19 days; No. S2, 20; No. C1, 23; No. C2, 24; No. S3, 24.5; Nos. C3, S4, and S5, 28; and Nos. C4 and C5, 31 days after the bacilli were injected. It will be seen from this that the splenectomized group (S) tended to die off a little sooner than the controls (C), but that the difference is not marked.

The necropsies reveal striking gross differences between the appearance of the pulmonary lesions in Group S and those of Group C, the most pronounced one being the type of lesion produced; the lungs of Group S are shot with discrete, firm miliary tubercles and float in water, those of Group C exhibit considerable consolidation and caseous pneumonia, and sink. Changes in the liver and kidney are not so marked in the gross specimens. The organs selected for microscopic examination are the lung, liver, and kidney, and the spleen when present. They have been fixed in Zenker's fluid and sectioned in paraffin, the sections being stained with hematoxylin-eosin, and with carbolfuchsin combined with Van Gieson's stain and with hematoxylin.

The Altered Histology in Splenectomized Animals.

Lungs.—As indicated by the gross examination, it is found that the tuberculous process in the lungs of Group S is essentially miliary,

[1] All operations were performed under ether anesthesia.

or conglomerate, in type, the tubercles are discrete, sharply delimited, and separated from one another by considerable expanses of practically normal lung tissue (Fig. 1). The tubercles also contain a great many polymorphonuclear amphophil cells, or their debris, which makes them stain very darkly as compared with those in the controls (Fig. 2). There are also fewer epithelioid cells in them, while those of the controls contain many. The Van Gieson sections appear to exhibit a trifle less reticulum in the tubercles of Group S than in those of Group C, but this is not striking. While there is much pneumonic exudate in the control lungs, composed chiefly of endothelial cells, with occasional areas of acute pneumonia, the lungs of the splenectomized animals show only occasional areas of intraalveolar exudate and usually these are made up principally of polymorphonuclear cells in various stages of disintegration. The overgrowth and metaplasia of the alveolar epithelium are more marked in Group C than in Group S, but they appear in sections from both groups and their intensity seem to depend largely upon the amount of reduction in the size of the air spaces, owing to exudation and to the blockage of bronchioles. As the lung sections of Group S approach those of Group C more closely in type, it is found that the epithelial overgrowth increases proportionately, whereas the reverse is true in the extreme types of either series. As there is more exudate in the less typical sections from Group S and in all the sections from Group C, the obvious conclusion is that the cutting off of the respiratory epithelium from its air supply must play an important part in bringing about the epithelial overgrowth.

The great thickening of the endothelial tissues of the pulmonary capillaries, their proliferative activity, and the increase in the number of free phagocytes noted in the experiments with carbon injections are not in evidence in this one. Indeed, the endothelium of the controls appears to be more active than that in the splenectomized animals. The air sacs of the lungs in Group C are crowded with phagocytes to a far greater extent than are those of Group S. This will be discussed later on.

The most striking feature noted in this experiment is the presence of much more numerous tubercle bacilli in the lesions of Group S than in those of Group C. While they are numerous in some of the

sections from the latter group, they are far more evenly distributed through the tubercles and exudate and many sections show much fewer bacilli than in any preparations from Group S. Those from the latter have the microorganisms concentrated in the tubercles among the polymorphonuclear cells in numbers which remind one somewhat of the appearance of the bacilli in lesions of leprosy, or the Johne-Frothingham disease of cattle. That there are more bacilli in the lungs of Group S than in those of Group C is indicated by the following experiment.

All the lung sections of both groups, stained with carbolfuchsin-hematoxylin, were mixed together in a tray and their identification marks hidden to avoid prejudice; they were then sorted out by microscopic examination into three lots, one containing many, one a moderate number of, and the third but few bacilli. Twenty-nine sections were examined, three from each rabbit save one (No. C2) for which only two slides were available. Of these lots, the first is found to be made up largely of sections from Group S, nine of them falling under this head. There are five sections from Group C in this lot. The second lot shows six Group S sections and one Group C; the third all Group C. This experiment was checked up by repetition with a second series of twenty-nine sections from the same blocks, but stained with Van Gieson-carbolfuchsin. The results are essentially the same. Averaging the two series of sections it is found that 70 per cent of the sections from Group S fall in the class of "many" tubercle bacilli, 30 per cent in that of "moderate numbers," and none in the "few." Of the sections from Group C, 30 per cent come under the heading "many," 22 per cent under "moderate numbers," and 48 per cent under "few." If the averages were based upon the carbolfuchsin-hematoxylin series alone the figures would not differ materially from these. The only marked exceptions to this rule are the six sections from Rabbit C5, all of which fall in the "many" class. This rabbit outlived all the others except Rabbit C4, whose lung sections fell mostly in the "few" category. It is difficult to explain why there should be this one striking exception.

The pulmonary lesions of Group S are, then, characterized by discrete tubercles containing many bacilli and polymorphonuclear amphophils. They are smaller and more compact than those of

Group C, in which diffuse lesions are the rule, fewer bacilli are present and these more diffusely distributed, and the tubercles themselves tend to be larger, not so dense, and more diffuse than in the splenectomized group. They contain fewer polymorphonuclear cells and their caseous centers are, therefore, more transparent. No differences in the appearance of the pulmonary endothelium distinguish the groups. The pulmonary epithelium shows more overgrowth and metaplasia in Group C than in Group S.

Liver.—In this organ, too, there is a marked difference between the distribution of the lesions produced in Group S and those in Group C, but the conditions observed in the lung are reversed; sections from Group S literally are riddled with almost contiguous tubercles (Fig. 3), running along the interlobular septa from Glisson's capsule; those from Group C contain the usual amount (Fig. 4), far fewer than in the splenectomized rabbits. Counting the tubercles to a low power (× 150) field, one finds on the average about ten tubercles in Group S (as many as seventeen were counted in sections from Rabbit S1), and four in Group C. Rabbit C5, whose lung sections showed so many tubercle bacilli, averaged but one tubercle to the low power (× 150) field in all three liver sections counted.

Kidney.—In this organ the tubercles were counted in each section, as there are but few, most low power fields containing none at all. They are situated usually in the cortex, and there are slightly more numerous lesions in the control series. In Group S they are small, immature, and poorly defined on the average, so that many may have been missed in searching; in Group C, however, they are larger, tend to undergo more extensive caseation, and are far more easily detected, so that it is possible that the actual number of tubercles per section in each series is essentially the same. The fact remains that the lesions are more advanced in the control animals.

Spleen.—Sections were made from all the spleens in Group C to determine the extent of involvement of this organ in the controls. They show tubercles in every instance, which vary in size and numbers, but the organ is always involved. Carbolfuchsin demonstrates large numbers of bacilli in these lesions, but it is to be noted that they seem to be in a poor state of preservation, staining indistinctly and appearing broken up.

Supplementary Experiment.

These results bring up several points that should be elaborated. (1) Are the pulmonary lesions in Group S small and discrete because of delayed development, or on account of the possibility that there has been an early proliferation of the endothelium—as seen in the carbon experiment—which has produced phagocytes that have removed the bacilli? (2) If the latter hypothesis be true, why is it that more bacilli are found in the less extensive type of pulmonary lesion observed in the splenectomized animals? (3) Why is the liver more extensively involved in the splenectomized rabbits? (4) Finally, why is the endothelial overgrowth more marked in Group C than in Group S, when we might expect exactly the reverse after considering the results of the carbon experiment?

In order to answer these questions, if possible, and to eliminate speculation, a second experiment was performed. Four rabbits, two splenectomized and two normal controls, were injected intravenously with 0.2 mg. per kilo of the same strain of tubercle bacilli employed in the first experiment. Two of the rabbits (Nos. C6 and S6) were killed at the end of 1 week, and two (Nos. C7 and S7) at the end of 2 weeks. The lesions produced correspond quite accurately with those observed in the first experiment; grossly there is little to be seen in the organs of the 1 week animals, excepting that the control shows an early pulmonary miliary tuberculosis. The 2 week rabbits show a rather diffuse miliary tuberculosis of the lungs in the control and a very discrete miliary distribution in the splenectomized animal; there are tubercles in the spleen of the control and in the livers of both; the kidneys show doubtful tubercles in the control. Microscopic examination brings out the following points.

Lungs.—The lungs of Rabbit C6 show scattered miliary tubercles not unlike those observed in the first experiment. Small numbers of well preserved tubercle bacilli are readily demonstrable. Sections from the lungs of No. S6, the 1 week splenectomized animal, show very few tubercles. Of four sections, one shows no tubercles whatever, the other three but one each, in every instance very immature. The pulmonary capillaries are crowded with polymorphonuclear

cells and the endothelium is universally thickened, nuclear knots composed of several nuclei being present here and there. The sections from the lungs of Rabbits C7 and S7, the 2 week animals, can be recognized by the naked eye as representing a more diffuse and a more discrete type of lesion respectively. Microscopically the control lung shows a type of lesion not unlike that of the splenectomized animals of the first experiment; the tubercles are still rather discrete, they have an admixture of polymorphonuclear cells in many instances, and there is, as yet, little pneumonic reaction, or intra-alveolar exudate, as compared with Group C of the first experiment. The alveolar walls are thickened by reason of a swelling of the capillary endothelium, which shows great proliferative activity and many mitotic figures. Many large phagocytes are invading the alveolar sacs and there forming syncytia. Tubercle bacilli are exceedingly difficult to demonstrate. The pulmonary sections from Rabbit S7 show discrete tubercles, separated by wider areas of comparatively normal lung tissue. The alveolar walls do not show the thickening or mitotic activity seen in the control. There are a great many polymorphonuclear leucocytes in the capillary lumina. Many tubercles show large numbers of these cells and their debris in their caseous centers, and in these one finds a large number of readily demonstrable tubercle bacilli. Reexamination of the more caseous centers of the tubercles in the lung sections of Rabbit C7 fails to demonstrate any bacilli, so that here too it may be said that tubercle bacilli are more readily found and are present in larger numbers than in the control, precisely as in the more advanced lesions of the first experiment. Apparently the lesion differs *ab initio* from that in the control lung and it may be concluded that the endothelium never has been proliferating so actively as in the carbon experiment. The lesions are merely retarded in development, and, although they contain more bacilli than those in the controls, they never become so diffuse. One cannot help associating this fact in some way with the presence of large numbers of polymorphonuclear cells that occur so regularly in the pulmonary capillaries of the splenectomized animals.

Liver.—Sections from the liver of Rabbit C6 show practically no tubercles, from that of No. S6 but few; in the former they may be

composed of a single syncytium, or a small group of endothelial cells and lymphocytes, while in the latter they are slightly larger. Tubercle bacilli may be found in both. At this stage the difference between the lesions in the splenectomized animal and those in the control is scarcely noted. In the liver sections from Nos. C7 and S7, the 2 week rabbits, there are a great many tubercles; those from the former average about seven, from the latter about twelve to the low power (× 150) field. More syncytia are found in the sections from No. S7 than in those from the control, and the liver is in an advanced stage of degenerative fatty infiltration. Tubercle bacilli are now difficult to demonstrate in both cases. In passing, it may be noted that the early, or immature tubercles present well preserved tubercle bacilli, both in the lungs and liver, while the larger, more mature tubercles do not show them so readily. The reason for this is not clear; it should be investigated more thoroughly.

Kidney.—Although the livers in this second series of experimental animals show little difference from each other, sections from the kidneys present marked distinguishing features; those from Nos. C6 and C7 present a greater number of more fully developed tubercles than do those from Nos. S6 and S7. In sections from No. C6 two or three tubercles are found in each, from No. C7 an average of eleven. The sections from No. S6 show no lesions at all, those from No. S7 an average of five per slide. There is, then, a marked inhibition in the development of tubercles in the kidneys of the splenectomized animals.

Spleen.—An examination of the spleens of Rabbits C6 and C7 throws no further light on the subject.

DISCUSSION AND SUMMARY.

If the spleen be removed from rabbits and tubercle bacilli be injected intravenously, it is found that the lesions produced differ materially from those observed in control animals; the lungs present the most marked contrast, the liver also shows a definite difference in the distribution of the tubercles, and the kidney lesions also differ in the two instances.

In discussing these facts we must answer, if possible, the four questions formulated above. It appears that the pulmonary lesions

in Group S are small and discrete because of some inhibitory factor that prevents the diffusion of the products of dead tubercle bacilli. The organisms, far from being killed, are more numerous and better preserved in this group, but their destructive action is localized. This seems to be connected with the presence of polymorphonuclear leucocytes, for these cells are more numerous in the lesions and more plentiful in the lumina of the pulmonary capillaries of Group S than they are in the controls. The conclusion to be drawn from this covers the answer to the second question. It is not the tubercle bacillus itself that produces the destructive changes, but the toxins liberated by the breaking down of its substance. The well known experiments of Hodenpyl and Armand-Delille, already referred to in this series of papers, show this to be true; dead tubercle bacilli, or even extracts of these organisms, will produce typical tubercles. Removing the spleen stimulates the production of polymorphonuclear leucocytes, as shown by Johnstone (1922). These cells may prevent the diffusion of split products from the bacilli by removing or neutralizing them.

The third question, as to why the liver is more affected after splenectomy than in normal controls, is more readily answered. Probably the spleen acts as a catch-basin for the bacteria; once removed it can no longer withhold them from the portal circulation and the liver receives a larger number than it would were this bacterial filter still operative. On the other hand, there may be an increase in the phagocytic activity of the endothelium of the sinusoids which might take up more bacteria under these changed conditions. Several investigators have claimed, recently, that there is an increased activity of the liver endothelium following splenectomy, their experiments being directed chiefly toward determining the fate of the erythrocytes.

Pearce (1918) in reporting the effects of experimental splenectomy in dogs, states that there are definite compensatory changes in the lymph nodes, in the form of an increased proliferation of endothelial phagocytes, and that the stellate cells of the liver sinusoids often show a similar compensatory increase in number. In both cases the cells are, apparently, formed *in situ* rather than transported to the organs. He says: "Such findings suggest the development of a compensatory function on the part of the lymph-nodes and possibly the liver," and suggests that, in times of stress "the stellate cells of the liver thus assume, in part at least, the function of destroying red blood-corpuscles by phagocytosis." Incidentally, he

presents an excellent discussion of the history and subject of splenectomy. Motohashi (1922) reports a great increase in the hemophagic power of the hepatic endothelium and an increase in the number of endothelial elements, after some 45 days following splenectomy in rabbits. Nishikawa and Takagi (1922) have observed similar phenomena with white rats, the Kupffer cells taking up erythrocytes in large numbers in splenectomized animals, whereas controls never show similar propensities on the part of these cells. It may be that different substances cause different reactions on the part of the hepatic endothelium.

Contributory Experiment.—A side experiment was performed with five rabbits, two splenectomized and three controls, into which uniform doses of pneumococci were injected intravenously. They all died of septicemia after a few days. The results of this experiment strengthen the foregoing conclusions materially. It was found that there were many polymorphonuclear amphophils in the pulmonary capillaries of the splenectomized animals and that there were numerous focal necroses in the livers. The controls showed much fewer polymorphonuclear cells in the lungs and no focal necroses in the livers, while the spleens were actively congested and inflamed. Otherwise the experiment was not of sufficient importance to warrant a separate report.

The question as to why the endothelium of the pulmonary capillaries shows no stimulation similar to that observed in the carbon experiment, but rather less activity than that of the controls, must be answered hypothetically for the present. With the carbon, comparatively huge amounts of foreign matter were injected repeatedly; here but one injection of a much smaller amount of suspended tubercle bacilli was administered. The resulting stimulus to the pulmonary endothelium would, therefore, differ materially in the two instances. In one there would be succeeding waves of stimulation following each injection of irritating foreign substance. In the other an entirely different sort of stimulus would result; the bacteria would be withdrawn from the circulation within an hour or two, judging by past experience, and would then multiply, to be cast off into the circulation in driblets, as the lesions containing them broke down. At least it can be said that there is a good theoretical reason for the difference in the endothelial reaction in the lungs of the two groups of animals.

CONCLUSIONS.

1. Splenectomy in rabbits, followed by the intravenous injection of tubercle bacilli, produces a changed distribution of these bacilli and of the lesions they cause.

2. The pulmonary lesions are fewer, more discrete, exhibit a larger number of polymorphonuclear leucocytes, and contain, as a rule, more tubercle bacilli than do those of controls.

3. The lesions in the liver are far more numerous and destructive in the splenectomized, than in the control animals.

4. The tubercles produced in the kidney do not show so marked a difference between the two groups as is noted in the lung and liver, but they are fewer and less well developed in the kidneys of the splenectomized rabbits.

5. The course of the disease is not affected materially by splenectomy, if anything it is rendered slightly more rapid; although the lung is less affected, the liver is so extensively invaded as to offset all the advantage that may have been gained by the decreased pulmonary involvement.

BIBLIOGRAPHY.

Armand-Delille, cited by Ranke, O., *Hist. u. histopath. Arb. Grosshirnrinde*, 1908, ii, 252.

Foot, N. C., *J. Exp. Med.*, 1923, xxxvii, 139.

Johnstone, E. M., *Arch. Surg.*, 1922, v, 159.

Motohashi, S., *J. Med. Research*, 1922, xliii, 419.

Nishikawa, Y., and Takagi, T., *Deutsch. med. Woch.*, 1922, xlviii, 1067.

Pearce, R. M., Krumbhaar, E. B., and Frazier, C. H., The spleen and anæmia, Philadelphia and London, 1918, 180.

Prudden, T. M., and Hodenpyl, E., *New York Med. J.*, 1891, liii, 697.

EXPLANATION OF PLATES.

PLATE 19.

FIG. 1. Low power photomicrograph of a section of the lung from a splenectomized rabbit (No. S4). Note the comparatively large amount of aerated tissue and the clumping of the lesions. Hematoxylin-eosin. × 16.

FIG. 2. Section from the lung of a control rabbit (No. C3) under the same magnification and with the same stain. The diffuse type of the lesion is obvious.

PLATE 20.

FIG. 3. Low power photomicrograph of a section from the liver of Rabbit S4. Note the large number of tubercles present. They are almost contiguous. Hematoxylin-eosin. × 100.

FIG. 4. Section from the liver of Rabbit C3. Only two large tubercles are present in this field; there are many giant cells. Hematoxylin-eosin. × 100.

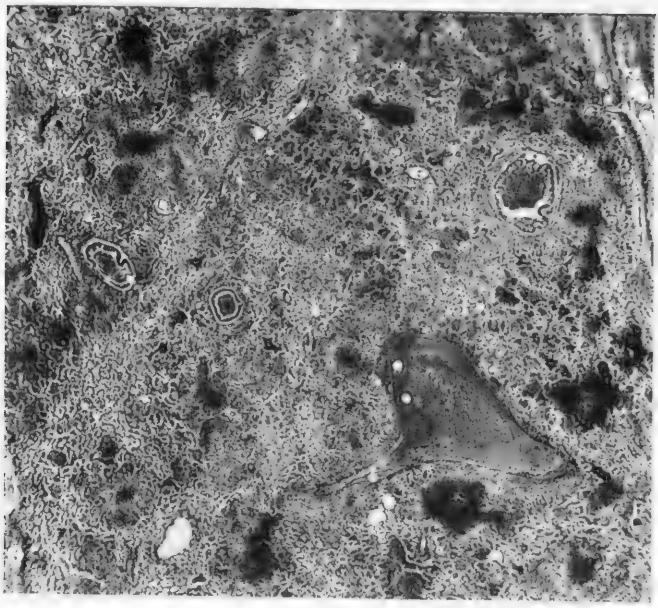


FIG. 2.

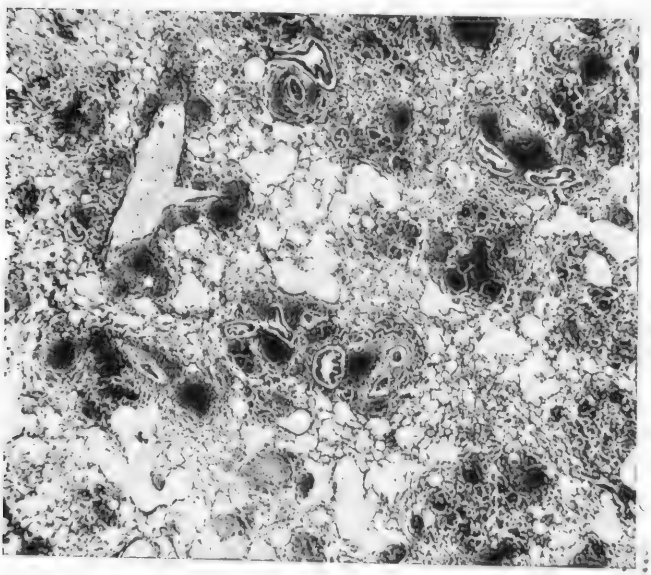


FIG. 1.

(Foot: Endothelial reactions. VIII.)

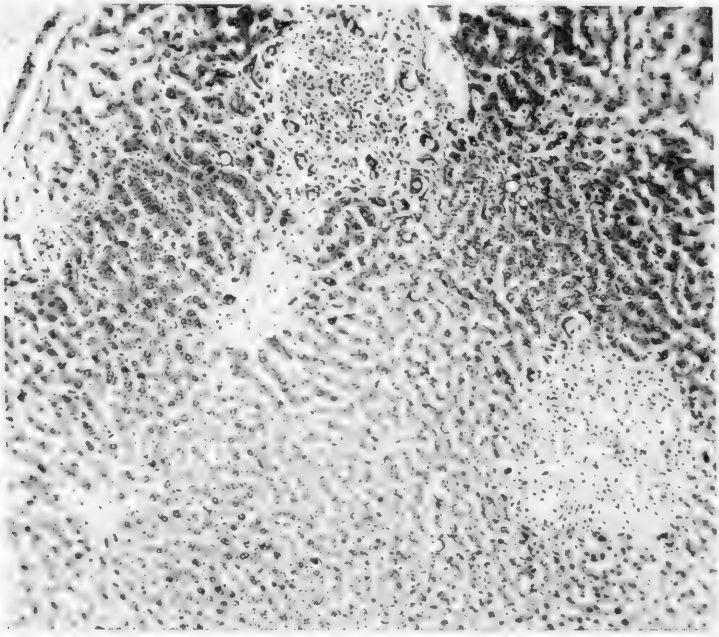


FIG. 4.

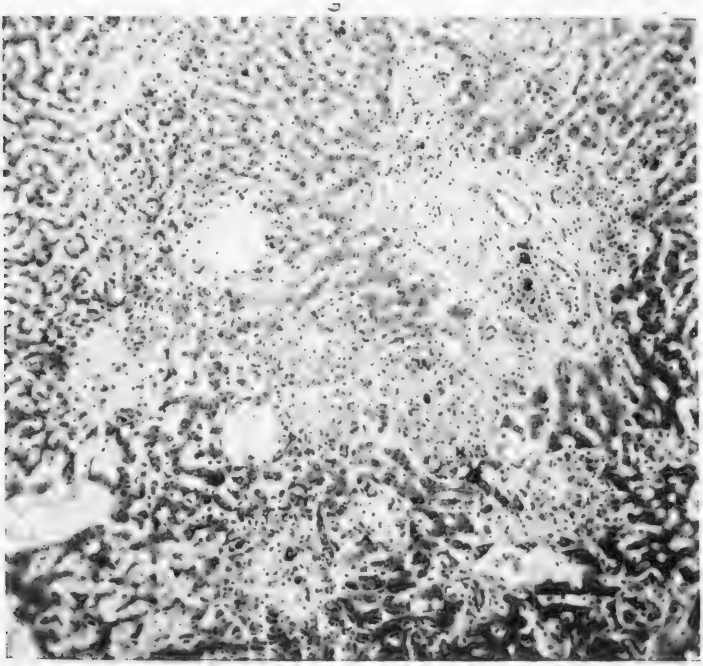


FIG. 3.

(Foot: Endothelial reactions. VIII.)

THE EFFECT OF PEPTONE ON THE PERIPHERAL CIRCULATION.*

By J. P. SIMONDS, M.D., and S. W. RANSON, M.D.

(From the Laboratories of Pathology and Anatomy† of Northwestern University Medical School, Chicago.)

PLATE 21.

(Received for publication, May 11, 1923.)

In a previous paper by one of us[1] it was stated that experiments were being carried out to determine (1) what influence, if any, the large mass of smooth muscle in the hepatic veins of the dog has on the fall in arterial blood pressure in anaphylactic and peptone shock; and (2) what other factors, if any, may be concerned in this reaction. The present paper pertains to the latter of these problems, and presents a study of the effect of peptone on the peripheral blood vessels.

The literature on this phase of anaphylactic and peptone shock is meager and the results reported are not uniform. Thompson[2] described a peripheral dilatation of the blood vessels of the dog, which he believed to be due to depression of the irritability of the vascular neuromuscular apparatus, rendering it irresponsive to vasoconstrictor influences.

Kaufmann[3] used the isolated rabbit's ear kept alive by perfusion with Ringer-Locke solution. Witte's peptone in concentration of 1:200 in this solution caused first a dilatation of the vessels, followed by a vasoconstriction which was of relatively long duration. He believed that the vasoconstriction represented the active phase of the effect of peptone because it disappeared if the vessels had previously suffered a severe irritation or if they were repeatedly subjected to the action of peptone. Pissemsky[4] studied the action of peptone on rabbit ears and

* This work was aided by a grant from the Fenger Memorial Fund.

† Contribution No. 106.

[1] Simonds, J. P., *Am. J. Physiol.*, 1923, lxv, 513.

[2] Thompson, W. H., *J. Physiol.*, 1896, xx, 455; 1899–1900, xxv, 1.

[3] Kaufmann, P., *Centr. Physiol.*, 1913–14, xvii, 724.

[4] Pissemsky, *Russk. Vrach*, 1915, xiv, No. 38; abstracted in *J. Am. Med. Assn.*, 1915, lxv, 2128.

obtained vasoconstriction with all concentrations and with any kind of peptone. Kondo[5] reports that peptone injected intravenously has a constricting action on the vessels of the legs and ears of rabbits, while it dilates the splanchnic vessels.

More recently Manwaring[6] has perfused the hind quarters of dogs with Locke solution containing 0.5 per cent filtered defibrinated blood from the same animal, under a perfusion pressure of 80 to 110 mm. of mercury. He found that the canine hind quarters, isolated by his method, usually showed a marked reduction in vascular tone, on control perfusion with Locke solution, but in one-third of his animals the vascular tone was fairly well preserved, at least during the initial stages of the perfusion. Manwaring found a uniformly increased perfusion flow from the hind quarters of his dogs varying from 10 per cent increase in the vascular atonic hind quarters, to 150 per cent increase in the vascular tonic hind quarters. At the height of the peptone reaction the rate of flow was identical with the perfusion rate with amyl nitrite. It appears that the reaction reached its maximum by the end of 1 minute, but with less tendency to recovery by the end of 8 minutes than in the isolated intestines similarly perfused.

It would seem, therefore, that most of the information available concerning the effect of peptone on the peripheral circulation has been obtained by perfusion experiments. Such procedures are handicapped by the complication that the tissues being perfused suffer from a coincident deficient oxygenation. Furthermore, in some of the experiments at least the perfusion pressure was maintained at a constant level. In our experiments these difficulties have been overcome, and they appear, therefore, to represent more accurately what actually takes place in the intact limb of a dog with the normal blood supply undisturbed.

Method.

In the present experiments now to be described the carotid blood pressure was taken in the usual manner with a mercury manometer, and the leg volume by means of a plethysmograph, the manometer and plethysmograph writing on the same kymograph. The plethysmograph used was one devised and previously described by one of us.[7] The technique differed from the usual method of plethysmographic study in two important respects: first, the instrument is so constructed that it obviates the necessity of making an air-tight joint

[5] Kondo, S., *Acta Scholæ Med. Univ. Imp., Kioto*, 1919–20, iii, 1.
[6] Manwaring, W. H., and Boyd, W. H., *J. Immunol.*, 1923, viii, 131.
[7] Ranson, S. W., and Wightman, W. D., *Am. J. Physiol.*, 1922, lxii, 392.

between the leg and the plethysmograph; and second, the peptone was injected in such a way that its effects on the vessels in the leg were not confused with changes due to vascular reactions in other parts of the body.

The dog is anesthetized with morphine and ether and the amount of anesthetic regulated so that the depth of anesthesia is constant throughout the experiment. The abdomen is opened and the right iliac artery isolated. The branches of this vessel are severed between double ligatures. The middle sacral artery is also ligated. The right iliac artery is doubly ligated and cut just above Poupart's ligament, and the free end brought out through an opening in the right flank. The abdominal incision is then closed. A pair of bulldog forceps is placed on the exposed iliac artery and there is inserted into the severed end and tied a large caliber blunt needle (for a Luer syringe) about the tip of which a small mass of solder has been fixed to prevent the needle from slipping out of the artery. The injections of peptone solution are made by attaching the syringe containing the solution to the needle, loosening the bulldog forceps, and forcing the liquid backward through the iliac artery to the bifurcation of the aorta whence it is washed into the left leg with the blood stream. By this method the peptone exerts its effects upon the vessels of the limb while they are supplied with normal blood in the normal manner, before it acts upon the vessels in any other part of the body. In this manner it is possible to determine the direct action of the peptone on the vessels of the leg uncomplicated, for a few seconds at least, by any general effect or by any special local effect that it may have upon any of the viscera.

The plethysmograph is essentially a flattened truncated cone. An air-tight rubber stocking is made of the same shape and size as the plethysmograph. The open end of the former is drawn over the open end of the latter and securely tied in place, the connection being made air-tight with a little beeswax in the groove around the open end of the instrument. The plethysmograph and stocking are filled with air and the left foot and leg of the animal are made to invaginate the small end of the stocking until the leg, covered by the invaginated stocking, is within the plethysmograph. The instrument is of such size that when the foot rests against the inside of the small end, the large end is at about the middle of the thigh. When supported by clamps and an iron stand with the leg held rigidly outward and backward no constriction is exerted on the femoral artery. When the instrument with the leg inside is in position warm water (40°C.) is introduced through a tube opening into the side while the air escapes through another tube attached to the small end of the instrument. A little air is allowed to remain in the upper end of the plethysmograph, and the tambour and connections contain air. With the instrument applied in this manner it is not only possible to record graphically the volume changes in the leg but the pulsations in the limb as well.

The peptone used in the experiments was the same as that employed in the experiments previously reported[1] dealing with the simultaneous changes in blood pressure in the carotid artery and jugular and portal veins. It was found to give uniformly a typical peptone shock in the dog.

RESULTS.

The results of the experiments are shown graphically in Fig. 1. The time relations between the changes in leg volume and carotid blood pressure are tabulated in Table I. Of the eight animals included in Table I, five suffered fatal peptone shock; two showed a non-fatal form, one of the animals receiving repeated injections of peptone recovering within a few minutes after each injection; and one dog which had been sensitized to normal horse serum was given 5 cc. of this serum, injected into the right iliac artery, in the same manner as the peptone.

From these experiments it is seen that the injection of peptone directly into the arteries of the hind limb of a dog causes an abrupt increase in the size of the leg due to vasodilatation. The increase in leg volume begins in from 5 to 9 seconds after the beginning of the injection of peptone and reaches its maximum in from 8 to 16 seconds after the beginning of the injection. The volume of the limb then becomes less almost as rapidly as it had increased and reaches its previous size in from 17 to 25 seconds after the beginning of the injection. In one animal the leg volume remained permanently slightly above its initial size. It was nearest its original volume 50 seconds after the beginning of the injection. In all the other dogs the leg volume showed a definite diminution, the minimum being reached in from 33 to 70 seconds from the beginning of the injection.

The arterial pressure in these animals showed, in almost all instances, a very slight fall of less than 10 mm. of mercury coincident with the beginning of the increase in leg volume. A permanent fall in arterial pressure occurred slightly later, or from 12 to 23 seconds after the beginning of the injection of the peptone, in all except one dog. This animal received three injections of peptone at short intervals and showed a beginning permanent fall in arterial pressure in 8, 9, and 8 seconds, respectively, after the beginning of the injections of the peptone.

It is thus seen that the permanent fall in arterial pressure in peptone shock in the dog begins after the leg volume has reached its maximum and is diminishing in size. The subsequent reduction in leg volume cannot, therefore, be due exclusively to the fall in arterial blood

TABLE I.

Time Relations between the Changes in Leg Volume and Arterial Blood Pressure.

Dog No.	Injection time.	Time after beginning of injection of peptone solution.					
		Beginning increase in leg volume.	Maximum leg volume.	Return of leg volume to normal level.	Minimum leg volume.	Beginning permanent fall in arterial pressure.	Minimum arterial pressure.
	sec.	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*
1	19	6	12	39	51	18	120
2	15	6	12	19	65	13	150+
3	12	9	14	25	40	16	40
4	12	7	10	17	70	13	120
5	15	5	8	17	33	23	90
6	18	8	13	50 (?)	50	12	28
7a	13	6	16	28	63	8	30
7b	17	6	14	28	60	9	37
7c	12	7	13	26	60	8	32
8	17	14	24	93	190	80	270

Nos. 1 to 5, fatal peptone shock.

Nos. 6 and 7, non-fatal peptone shock. 7a, b, and c represent injections into the same animal at short intervals after the leg volume and arterial pressure had returned to normal following the preceding injection.

No. 8, non-fatal anaphylactic shock. Dog immunized with normal horse serum and injected with 5 cc. of the serum after 3 weeks.

pressure. It is evident, furthermore, that the permanent fall in arterial pressure is not the result of peripheral dilatation, for this has been shown to be of very short duration, and has started to disappear before the arterial pressure begins to show its characteristic fall.

The decrease in leg volume below its original level shows that the blood does not accumulate in the peripheral vessels in peptone shock and must, therefore, be impounded elsewhere in the body.

With suitably regulated doses of peptone the results described above can be repeated several times in the same animal (see Dog 7, Table I). The arterial pressure and leg volume are allowed to return to normal before the injection is repeated. In this respect, therefore, peptone shock differs from anaphylactic shock. The fundamental physiologic reaction appears, however, to be the same in both conditions.

The single instance of anaphylactic shock in this series indicates that changes occur in that condition similar to those in peptone shock (Dog 8, Table I). The peripheral dilatation was less marked in this dog than in those showing peptone shock. Furthermore, the fall in arterial pressure was later in manifesting itself than in other dogs in which the serum to which they had been sensitized was injected into the femoral artery instead of into the iliac artery.

The vasodilatation produced by the intraarterial injection of peptone may be due to the presence of a small amount of histamine as an impurity.

It has been shown by Abel and Kubota[8] and by Hanke and Koessler[9] that Witte's peptone contains appreciable amounts of this toxin, in one sample as much as 0.0335 mg. per gm. Since the intraarterial injection of 0.025 mg. of histamine is sufficient to produce a maximum vasodilatation in the hind leg of a dog[10] it is obvious that the amount of this substance in peptone is sufficient to account for its dilator action. Moreover, peptone, as shown by Kaufmann[3] and Kondo[5] and confirmed by us in one experiment, has a vasoconstrictor instead of a vasodilator action in the rabbit. Since histamine also causes vasoconstriction instead of vasodilatation in the rabbit[11] the action of peptone on the peripheral vessels seems to be identical with that of histamine. Although it is possible that the transient vasodilator action of peptone may be due to contamination with histamine, Hanke and Koessler[9] have shown that histamine and peptone shock are not identical, since a typical peptone shock is obtained by the injection of histamine-free peptone.

[8] Abel, J., and Kubota, S., *J. Pharmacol. and Exp. Therap.*, 1919, xiii, 243.

[9] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 567.

[10] Ranson, S. W., Faubion, L. R., and Ross, C. J., *Am. J. Physiol.*, 1923, lxiv, 311.

[11] Dale, H. H., and Richards, A. N., *J. Physiol.*, 1918–19, lii, 110.

SUMMARY.

1. Injection of a solution of peptone directly into the artery of the leg of a dog is followed by an abrupt increase in leg volume. This is of short duration and is quickly followed, usually in less than $\frac{1}{2}$ minute, by a decrease in the volume of the limb below that previous to the injection.

2. The phases of the leg volume curve bear a fairly definite relation to the changes in arterial pressure.

3. These results, therefore, indicate that the long continued low arterial pressure in peptone shock, and probably in anaphylactic shock, may be hastened but is not maintained by peripheral dilatation, and suggest that there is an impounding of the blood in some other part of the body.

EXPLANATION OF PLATE 21.

FIG. 1. Arterial blood pressure (*A*) and leg volume (*L V*) of Dog 7 in Table I. Time marked in seconds.

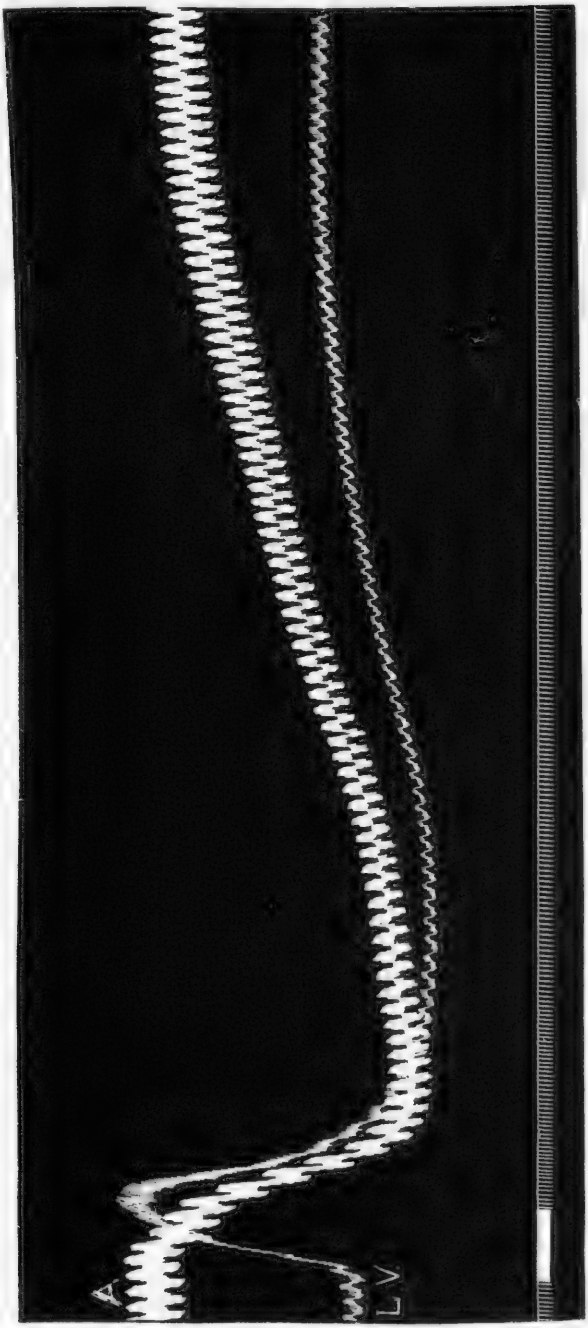


FIG. 1.

(Simonds and Ranson: Effect of peptone on peripheral circulation.)

PROTECTION AGAINST TYPHOID-LIKE INFECTIONS BY VACCINATION.

An Experimental Study.

By HENRY J. NICHOLS, M.D.,

Major, Medical Corps, U. S. Army,

and CLARENCE O. STIMMEL,

Captain, Medical Administrative Corps, U. S. Army.

(*From the Laboratory Division of the Army Medical School, Washington, D. C.*)

(Received for publication, May 25, 1923.)

The basic fact that infections with the typhoid bacillus can be prevented by vaccination is, of course, well established by clinical evidence. It is also supported, theoretically, by a certain amount of experimental evidence. Problems still remain, however, in regard to the degree and duration of immunity, the best kind of vaccine, its keeping qualities, and the best method of administration. The experimental solution of these problems has been delayed by the impossibility of reproducing a true typhoid infection in the common laboratory animals. In the absence of a real protection test, efforts have been made to settle many questions by a quantitative determination of the antibody content of the serum of vaccinated animals. This method has been of practical value in some directions, but of course, in general, is inferior to an actual protection test. As an illustration of the limitations of the antibody method, it may be stated that typhoid vaccine, 10 years old, produces almost as good an agglutination titer in the rabbit as fresh vaccine, but its protective value is much less.

Another method of testing immunity has been the use of typhoid intoxication in the common laboratory animals. By intravenous or intraperitoneal injections of living bacilli, differences in toxicity can be determined in different strains of the typhoid bacillus and protection by vaccination can be demonstrated. This method also has some value, but it is highly artificial, as the doses are usually enormous and

there is no true septicemia. The same is true of the Gay method of using gall bladder infections as a test of immunity.

In recent years the discovery of the natural occurrence of paratyphoid infections in animals as well as in man has opened the way for actual protection tests with this group of organisms. Clinically, paratyphoid vaccination is as effective as typhoid vaccination and there seems to be no reason why the information obtained from vaccination with the paratyphoid group of bacilli should not be applied to the whole Salmonella group. This method has recently been used, experimentally, by Besredka (1) in developing his ideas on local immunity and by Flexner and Amoss (2), Webster (3), and Lynch (4) in epidemiological work among mice. We have also used this method for several years in attempting to solve some of the problems mentioned above, in connection with the manufacture of large amounts of triple typhoid vaccine at the Army Medical School. Even this method, in our hands, is somewhat artificial, but the results are none the less definite and valuable.

Considerable work along the same lines has been done with other groups of organisms, especially the Pasteurella group. The most extensive recent work has been that of Harvey (5), who worked with pigeons and after vaccination with *B. avisepticus*, gave intravenous infecting doses. He concludes (*a*) that an avirulent organism protects as well as a virulent one, (*b*) that multiple small doses protect better than one large dose, (*c*) that there is some group and nonspecific protection, and (*d*) that vaccine does not deteriorate with age or temperature up to 6 to 9 months.

Our work started in 1921 with a spontaneous and severe epidemic among guinea pigs, which furnished us with a picture of the natural disease. The causative organism was the "mutton" strain of *Bacillus aertrycke*, which can be differentiated from the human paratyphoid B bacillus only by absorption of agglutinins.

Method.

Male guinea pigs of approximately the same weight were selected and kept under observation for about a week. They were then vaccinated, subcutaneously, at 8 to 10 day intervals and 15 days after the last dose of vaccine were given the infecting dose, also subcutaneously.

This dose was about 400 million organisms. The virulence was such that the controls died in 6 to 10 days. Each animal that died was autopsied and the lesions were noted and cultures were made from the spleen, liver, gall bladder, and heart's blood. Surviving animals were chloroformed and autopsied after 30

days and similar cultures were made. The organisms were identified by agglutination. A few surviving animals showed possible residua of infection with negative cultures, and were considered not infected. Precautions were taken to avoid sudden drop in animal room temperature which is often followed by a number of deaths from non-specific pneumonia.

Careful adjustment of the virulence of the virus is necessary and several preliminary series were lost through failure to observe this point. In one series, all the vaccinated animals died as well as all the controls, although the vaccinated animals lived longer and had less extensive lesions. In another series, none of the controls became infected. It was finally found that by making blood cultures from sick animals and by transplanting to blood broth, the virulence could be maintained at a fairly fixed point. The infecting doses were made from 18 hour blood broth cultures. In each experiment, as far as possible, four animals were used, with four normal controls, and four controls vaccinated with fresh, full strength, saline vaccine, 1 cc. of which contained about 1,000 million organisms.

An attempt was made to imitate natural conditions by feeding bacilli and by inoculation of the alimentary tract by means of a medicine dropper and fairly good results were obtained in one series, but this method was given up for the subcutaneous one, on account of more certainty of infection.

The animals all developed a local lesion at the site of inoculation consisting of a suppurating nodule which usually broke down and discharged. In normal animals this lesion was large and edematous; the animals appeared sick and usually had a conjunctivitis. In protected animals, the lesion was sharply localized and no systemic reaction was noted. The specific internal lesions in non-protected animals consist of an enlarged and granular spleen and liver, an infected gall bladder, and usually a pneumonia.[1]

Control Experiments with Full Strength Fresh Saline Vaccine.

TABLE I.

Group.	No. of animals.	Protected.	Not protected.
Control	12	0	12
Vaccinated (2,500 million)	11	11	0

These results were all clean-cut; the controls all died of the acute disease and all cultures were positive except in one instance of death on

[1] The experimental stock was obtained by courtesy of the United States Department of Agriculture and was free from natural infection.

the 27th day in which the organisms were found only in the gall bladder contents (Table I). None of the vaccinated animals showed any signs of disease except locally. The vaccinating doses were 500, 1,000, and 1,000 million bacilli subcutaneously at 8 day intervals, a total of 2,500 million organisms. This technique affords, therefore, a definite method by which it is possible to secure reliable data on various questions.

Relation of Dose of Vaccine to Protection.

The doses used to vaccinate animals are usually the same as for adult human beings and of course are enormously larger than comparative body weight would call for. The infecting dose, however, is also much larger than the proportionate dose for man. But the effect of different sized doses of vaccine is instructive.

TABLE II.

Total No. of organisms injected.	No. of animals.	Protected.	Not protected.
625 million.	4	3	1
300 "	3	1	2
25 "	3	1	2

The vaccine was given in the regular way in three doses of 0.5, 1.0, and 1.0 cc. The dose in the third experiment, is the adult human dose reduced approximately to body weight for the guinea pig. Only one out of three animals was protected (Table II). All the animals in this series, which are marked not protected, died of the disease, with positive cultures. It is seen again that the protection varies with the number of organisms injected.

Effect of Age of Vaccine on Protection.

The question of the keeping power of prophylactic vaccines is of importance, economically, as well as immunologically. Attempts to settle this question by antibody production are not convincing. As will be seen from Table III, there is a falling off of protection after 8 months and we believe that this is about the time limit for this kind of vaccine.

TABLE III.

Age of vaccine.	No. of animals.	Protected.	Not protected.	
mos.				
Fresh.	11	11	0	100 per cent protection.
4	4	4	0	
7	3	3	0	
8	4	4	0	
10	4	3	1	66 per cent protection.
11	4	2	2	
14	4	3	1	

All the animals marked not protected died of the disease, except one of the 11 months series, which survived but on autopsy showed lesions, with organisms, in the spleen and gall bladder. The vaccine was kept under conditions which resembled those of actual use, part of the time at room temperature and part of the time in the ice box, with occasional shaking. It is seen that vaccine up to 8 months old gave complete protection in twenty-two animals. Vaccine 10 to 14 months old protected only eight out of twelve animals, a falling off of one-third in protective power.

Effect of Different Kinds of Vaccine.

The exact kind of vaccine has, of course, been the subject of much work and discussion. Our results with several different kinds are given in Table IV.

TABLE IV.

Vaccine.	No. of animals.	Protected.	Not protected.
Lipovaccine (2,500 million)	5	3	2
Resuspended vaccine	3	3	0
Supernatant fluid	4	2	2
Sensitized vaccine (killed)	4	3	1
T. A. B. vaccine	3	3	0

Lipovaccine.—The animals were given a single dose in oil equal to the divided saline doses. All the animals survived, but two on autopsy showed specific lesions of the spleen and gall bladder with positive cultures.

Resuspended Vaccine.—The vaccine was allowed to stand for about 14 days in 5 cc. ampules until the supernatant fluid was crystal-clear. This fluid was pipetted off and the sediment was resuspended in an equal amount of salt solution. The results were as good as with the original vaccine.

Supernatant Fluid.—The supernatant fluid, obtained as stated above, was used as a vaccine. This fraction has some protective power, but one animal died of the disease and one of three surviving animals was infected.

Sensitized Killed Vaccine.—This was apparently not so effective as the original vaccine, as one animal died of typhoid disease. Four animals, given a living sensitized vaccine, all died of the disease contracted from the first dose of vaccine.

T. A. B. Vaccine.—This group vaccine protected fully. The total Para B dose was 1,250 million, Para A, 1,250 million, and typhoid, 2,500 million.

We also have some partial data which tend to show that detoxicated (6) and filtered vaccines do not protect so well as the original vaccine. Our work on vaccination by mouth (Besredka) is too incomplete to be discussed, but Webster (3), working with the same organism in mice, found no evidence of local immunity.

DISCUSSION.

The experimental work cited above supports the following conclusions and general rules for practise in prophylactic vaccination against the typhoid group of infections. It may appear that some of this work is superfluous, as several of the conclusions reached are already accepted, but it seems desirable to record all the actual evidence obtainable.

1. Acquired immunity is a variable factor and depends partly on the dose of vaccine. A high immunity can be obtained by a sufficient vaccination but can be overcome by a large enough infecting dose. Repeated doses give better immunity than a single dose. It is probably also true that frequent courses give the greatest protection, which is probably the reason for the freedom of the Army from these infections. According to regulations, vaccination is given every 3 years for three courses. But not infrequently records are lost and vaccinations are repeated oftener than called for.

2. A relatively fresh vaccine gives better protection than an old one; the period of maximum efficiency is about the first 8 months.

3. Plain saline vaccine is superior to lipovaccine, sensitized vaccine, or some fractions. Vaccination with sensitized living vaccine produced the disease instead of protecting.

The principal point of interest in this connection is probably in regard to the use of lipovaccine. It is not intended to discuss the subject fully here, but it may be said that the use of lipovaccine was discontinued in the Army, primarily, on account of the difficulty of making a sterile product on a large scale. When methods were available for making a sterile vaccine, lipovaccine was not reissued because of doubts as to its protective power. As judged by the production of antibodies, it is distinctly inferior to saline vaccine (7). Judged by the present method it undoubtedly gives some protection and in an emergency might be used on account of the definite advantages of a single dose. The best protection, however, is afforded by multiple doses of a saline vaccine.

The subject of group and non-specific immunity has come to the front recently. A partial non-specific protection has been demonstrated experimentally by Harvey (5), by Clark (8), and by others. According to the results given above, a group vaccine proved as effective as the specific vaccine. It should be remembered, however, that the human Para B bacillus is very closely related to *Bacillus aertrycke*. In our Service, on the Mexican Border before the War, typhoid vaccine, alone, gave no adequate protection against infections with Para A and B. During the war, the same fact was demonstrated in the British Army and a paratyphoid vaccine was insisted on and proved effective.

SUMMARY.

1. A natural infection of guinea pigs with the "mutton" strain of *Bacillus aertrycke* was used to test the protective power of vaccination against the typhoid group of infections.

2. Under the conditions of the experiment, complete protection was secured by vaccination with full strength fresh saline vaccine, while 100 per cent of deaths occurred among the controls.

3. The immunity acquired is variable and depends on the number of organisms injected.

4. Vaccine kept 10 to 14 months gave less protection than vaccine 8 months old and under.

5. Saline vaccine was more effective than lipovaccine, sensitized vaccine, or supernatant fluid vaccine.

6. Resuspended vaccine was as effective as the original vaccine.

7. In one experiment, group vaccine, made of typhoid Para A and Para B bacilli, was as effective as the original specific vaccine.

BIBLIOGRAPHY.

1. Besredka, A., *Ann. Inst. Pasteur*, 1919, xxxiii, 301, 557, 882. Masaki, S., *Ann. Inst. Pasteur*, 1922, xxxvi, 399.
2. Flexner, S., *J. Exp. Med.*, 1922, xxxvi, 9. Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25, 45.
3. Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71, 97; 1923, xxxvii, 231, 269.
4. Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.
5. Harvey, W. F., and Iyengar, K. R. K., *Indian J. Med. Research*, 1921–22, ix, 726, 730, 736. Harvey, W. F., *Indian J. Med. Research*, 1921–22, ix, 740. Harvey, W. F., and Iyengar, K. R. K., *Indian J. Med. Research*, 1922, x, 190, 192; 1923, x, 990, 1147.
6. Coppinger, F. R., and Robertson, R. C., *J. Roy. Army Med. Corps*, 1922, xxxviii, 243.
7. Gay, J. N., *J. Infect. Dis.*, 1921, xxix, 417.
8. Clark, P. F., Zellmer, C. E., and Stone, H. W., *J. Infect. Dis.*, 1922, xxxi, 215.

OXIDASE REACTION OF VARIOUS GROUPS OF BACTERIA.

By LLOYD D. FELTON, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 24, 1923.)

The oxidases of both animal and plant tissue have been extensively studied; they are found almost universally in all forms of life and exert undoubtedly an important function in the oxidation in living cells. The constitution of these enzymes and the mechanism of their action are not understood. They have been defined by Portier[1] as "agents found in life which have the power to oxidize certain substances in the presence of oxygen in gaseous or dissolved state." Duclaux[2] states that: "Oxidases are substances which at ordinary temperatures and under physiological conditions carry oxygen rapidly to materials upon which, without the intervention of oxidases, ordinary oxygen would act very slowly." The different oxidases are usually classified on the basis of the substance which is oxidized; that is, a tyrosinase is an oxidase which oxidizes tyrosine; an aldehydase one which oxidizes an aldehyde, etc. Whatever their constitution or mechanism of action, it is understood that oxidases are known to change the so called inactive molecular oxygen into oxygen in the active state, suitable for chemical union, and that without the aid of these agents as an expression of vital activity oxidation takes place very slowly, if at all. Hence, the importance of any study that may furnish a better insight into a chemical reaction so important to all forms of life.

From a survey of the literature it is evident that few investigators have studied or commented on this activity in the case of the ordinary microorganisms, though great attention has been focused upon the oxidases of animal and plant tissue.

[1] Portier, Les oxydases dans la séries animale, leur rôle physiologique, Thèse de Paris, No. 63, Paris, 1897.

[2] Duclaux, E., Traité de microbiologie, Paris, 1899, ii, 565–584.

Schönbein,[3] as early as 1855, made a study of fungi and found positive oxidase activity. Roux,[4] in 1899, demonstrated the existence of an oxidase in a culture of *B. coli*. Yeast was shown to be capable of producing oxidases by Tolomei,[5] Effront,[6] Buchner,[7] Grüss,[8] and Issajew.[9] Dietrich and Liebermeister[10] found this ferment in pustules. Lehmann and Sano,[11] in a study of thirty microbic species, detected the presence of a tyrosinase in three; namely, *B. putidum*, *B. phosphorescens*, and *Actinomyces chromogens*. Kastle's monograph on oxidases[12] provides an excellent bibliographic source and historical presentation of the development of knowledge concerning oxidases.

While estimating the bactericidal action of certain compounds of the triphenylmethane leuco bases, I observed that some of them were changed into their corresponding dyes in the presence of growing pneumococci, a chemical reaction not actuated by individual strains of *Streptococcus hæmolyticus*, *Staphylococcus aureus*, meningococcus, or *Bacillus typhosus*. The oxidative phenomenon was first noted with *p*-aminoleucomalachite green when pneumococci were grown on whole horse blood. Control tubes consisting of whole defibrinated horse blood and the leuco compound remained colorless for a period of 96 hours. With the pneumococcus a deep purple color developed, the shade being that of *p*-aminomalachite green. Obviously, the dye was

[3] Schönbein, C. F., Ueber Ozon und Ozonwirkungen in Pilzen, *Phil. Mag.*, 1856, ii, series 4, 137; Ueber die Selbstbläuung einiger Pilze und das Vorkommen von Sauerstofferregern und Sauerstoffträgern in der Pflanzenwelt, *Verhandl. Naturf. Ges.*, 1855, i, 339; Ueber Ozon und Ozonwirkungen in Pilzen, *J. prakt. Chem.*, 1856, lxvii, series 3, 496.

[4] Roux, Sur une oxydase productrice de pigment, sécrétée par le coli-bacille, *Compt. rend. Acad.*, 1899, cxxviii, 693.

[5] Tolomei, G., Sopra un fermento solubile che si trova nel vino, *Atti Real. Accad. Lincei, Rend. sc. fis., mat. e. nat.*, 1896, v, series 5, 52.

[6] Effront, J., Enzymes et leurs applications, Paris, 1898; English translation by S. C. Prescott, New York, 1902.

[7] Buchner, E., Ueber zellenfrei Gährung, *Ber. chem. Ges.*, 1898, xxxi, 568.

[8] Grüss, J., Über Oxydaseerscheinungen der Hefe, *Woch. Brauerei*, 1901, xviii, 310, 318.

[9] Issajew, W., Ueber die Hefeoxydase, *Z. physiol. Chem.*, 1904, xlii, 132.

[10] Dietrich, A., and Liebermeister, G., Sauerstoffübertragende Körnchen in Milzbrandbacillen, *Centr. Bakt., Ite Abt., Orig.*, 1902, xxxii, 858.

[11] Lehmann, K. B., and Sano, Über das Vorkommen von Oxydationsfermenten bei Bakterien und höheren Pflanzen, *Arch. Hyg.*, 1908, lxvii, 99.

[12] Kastle, J. H., The oxydases, *Bull. Hyg. Lab., U. S. P. H., No. 59*, 1909.

oxidized from its leuco base by the activity of the growing pneumococci.

This observation presented an opportunity for studying the problem of the oxidase activity of bacteria by means of a simple technique. Leucomalachite green had been used for the detection of blood by Adler and Adler[13] and later by Buckmaster[14] in a study of pseudoperoxidase reaction between hemoglobin and its derivatives. The compound used by them was the leucotetramethyldiaminotriphenylmethane, whereas the *p*-aminoleucomalachite green made use of by a simple technique in the present study is the 4, 4,-bis dimethylamino-4-aminotriphenylmethane. We wish to present here, first, a study of the pneumococci in relation to the oxidase activity under various conditions of growth, and, second, an inquiry into the oxidases of other microbic species as determined by this indicator.

Technique.

Solution of the indicator is made by dissolving a weighed portion of the leuco base in dilute hydrochloric acid, which is added drop by drop on the dry chemical. After dissolving, water is added to make a 1:100 concentration and the excess of acid neutralized by dilute sodium hydroxide, enough alkali being used to produce just a slight opalescence. Sterilization can be effected by boiling, or autoclaving under nitrogen, or by means of a Berkefeld filter. For practical purposes, it was found sufficient to boil in an excess of hydrochloric acid, and then neutralize with sterile sodium hydroxide (N) after the solution became cool. The amount of leuco base used in the test represented a final dilution in the media of 1:10,000.

The usual method was to pipette 2 cc. of medium into a 10 mm. tube, add, in turn, 0.1 cc. of a 1:500 concentration of leuco base and then 0.1 cc. of an 8 hour culture of the bacteria in question. The inoculated tubes were incubated for varying lengths of time at 37.5°C. Controls of both medium and indicator were always run.

Conditions Which Influence the Reaction with Pneumococcus.

As stated above, the reaction was first noted when whole defibrinated horse blood was used as medium. Serum was found to give as suitable conditions as whole blood, the one requirement for both being

[13] Adler, O., and Adler, R., *Z. physiol. Chem.*, 1904, xli, 59.

[14] Buckmaster, G. A., The pseudo-peroxydase reaction between hæmoglobin, its derivatives and the leucobase of malachite green, *J. Physiol.*, 1908, xxxvii, p. xi.

that they be fresh. Contrarily, plain broth or meat infusion (plain broth with peptone) and 1 per cent peptone solution, each having a pH of 7.8, prevented the oxidation of the indicator by pneumococci. The difference is striking and perhaps significant. Pneumococci growing in serum cause oxidation of *p*-aminoleucomalachite green, whereas growth, seemingly as abundant on the broth, meat infusion, and peptone media as on serum, produced not the slightest oxidation of the indicator. The reaction is unique in that it appears to require something in fresh animal tissue (fresh serum) as a substrate. Although it is not our purpose to explain the reaction in this communication, the following experiments were performed in quest of a possible reason for the difference in action between media with and without serum.

Experiment 1.—Variation in the concentration of serum, plain broth, and meat infusion from 100 to 10 per cent, in gradients of 10.

Experiment 2.—Variation in the concentration of meat infusion, peptone solution, and plain broth in a quantity of horse serum that rendered possible the oxidation of this indicator.

Experiment 3.—Variation of peptone solution and meat infusion in guinea pig serum.

The results of these experiments are given in Table I. The leuco compound is not oxidized by growing pneumococci in any concentration of the media without serum, while complete oxidation occurs in serum in as high a concentration as 70 per cent. In undiluted serum and in 80 and 90 per cent serum, definite inhibition of the oxidation reaction is manifest. The results in these dilutions indicate the existence of a reducing potential, perhaps an antioxidase, which counterbalances the oxidation by the microorganism. Experiment 2 emphasizes the reciprocal relationship; that is, at a definite concentration of broth and meat infusion media, in a quantity of serum in which a positive reaction would normally be obtained, no oxidation of the indicator is observed. It might be thought that the meat infusion, broth, and peptone media have a destructive action on the oxidizing enzymes of the serum; indeed, this mechanism might be explanatory of the difference between the action of these media and serum, were it not for the fact that undiluted serum inhibits or prevents the reaction in a similar way.

By chance, guinea pig serum was used in one of the experiments instead of horse serum, and the impression was gained that oxidation proceeded much more readily in guinea pig serum than in horse serum. To study the question further, Experiment 3 was carried out. The

TABLE I.

Influence of Different Media on Oxidase Production by Pneumococcus.

Medium.	Diluent.	Percentage of dilution. Medium. 100	90	80	70	60	50	40	30	20	10	0
		Diluent. 0	10	20	30	40	50	60	70	80	90	100
Meat infusion (pH 7.8).	Buffer salt (pH 7.8).	0	0	0	0	0	0	0	0	0	0	0
Plain broth (pH 7.8).	" " (pH 7.8).	0	0	0	0	0	0	0	0	0	0	0
Fresh horse serum.	" " (pH 7.8).	1	1	2	4	4	4	4	4	4	4	0
20 per cent fresh horse serum.	20 per cent meat infusion.	4	3	2	2	1	1	Tr.	0	0	0	0
20 " " " " "	20 per cent of 1 per cent peptone solution.	4	3	2	1	0	0	0	0	0	0	0
20 " " " " "	20 per cent plain broth.	4	3	2	1	0	0	0	0	0	0	0
20 " " " guinea pig serum.	50 per cent meat infusion.	4	4	4	4	4	4	4	4	3	2	0
20 per cent fresh guinea pig serum.	50 per cent of 1 per cent peptone solution.	4	4	4	3	2	1	Tr.	0	0	0	0

The figures 1, 2, 3, and 4 represent respectively 25, 50, 75, and 100 per cent oxidation as judged by the intensity of the color of the indicator. 0 means no visible oxidation.

All tubes contained 2 cc. of medium and 0.2 cc. of 1:500 *p*-aminoleucomalachite green.

Observation made after 48 hour period of growth.

results show definitely that guinea pig serum possesses the essential elements for the oxidation of the indicator by pneumococci to a greater degree than horse serum. If undiluted meat infusion is used as a basis for comparison it is seen that a 1 per cent mixture of meat infusion

with a constant amount of horse serum inhibits the reaction; whereas, 40 per cent of meat infusion is necessary to cause the same inhibition in guinea pig serum. Because of the result of the last experiment, sera from seven different animal species, under as nearly the same conditions as practicable, were studied. The experiment with each specimen was repeated three times with sera of rat, guinea pig, rabbit, horse, man, cat, and chicken, with the following results. In order of suitability for the oxidation reaction, the general grouping was: rat, guinea pig, and rabbit best; horse next best; man, cat, and chicken least suitable. There exists the possibility that the variation in the sera from different animal species may be due to the presence of hemoglobin, though reasonable care was exercised in securing the specimens of sera. The animals, with the exception of the horse, were bled from the heart by means of a syringe with a new needle, both of which had been washed out with normal physiological salt solution prior to use.

In addition, it has been noted that hemoglobin influences the reaction; a small amount is stimulatory, a larger quantity inhibitory. Oxygen is essential for the reaction itself, no change taking place under anaerobic conditions. However, if air is admitted to an 8 hour culture which has been grown anaerobically in suitable medium, oxidation takes place almost immediately. H ion concentration between the limits of pH 6 and 7.8 increases the rate of oxidation in direct ratio to the H ion concentration,—the more acid the media, the more rapid the reaction. Virulence of the pneumococci does not seem to cause any change in the degree or rate of oxidation of the indicator. During the course of another investigation, the virulence of a strain of pneumococcus was so altered that the minimum lethal dose of various cultures for mice was as follows: 2, 1,800, 46,000, 120,000, and 3,000,000 organisms. The cultures were tested in varying amounts of meat infusion in a constant quantity of guinea pig serum. No difference in the reaction was discernible. It was also found that heating fresh serum for 30 minutes at 55°C. made it more suitable for the reaction, in part destroying the inhibiting power of undiluted serum. A concentration of 1 per cent dextrose in serum was found to increase the rate of oxidation by pneumococci.

Reaction with Different Microbic Species.

In applying the test to other species of microorganisms, we have made use of conditions for oxidase production which were optimum for pneumococci; namely, 10 per cent fresh horse serum, 10 per cent fresh horse serum with the addition of 1 per cent dextrose, and 10 per cent

TABLE II.

Failure of the Oxidase Reaction.
Miscellaneous Group of Bacteria.

Organism.	No. of strains.	Medium pH 7.8.			
		10 per cent horse serum.	10 per cent horse serum with 1 per cent dextrose.	10 per cent guinea pig serum.	Plain broth.
Staphylococcus aureus.	6	0	0	0	0
" *albus.*	4	0	0	0	0
Meningococcus.	14	0	0	0	0
B. typhosus.	3	0	0	0	0
" *paratyphosus* A.	2	0	0	0	0
" " B.	3	0	0	0	0
" *coli communis.*	5	0	0	0	0
" " *communior.*	2	0	0	0	0
" *dysenteriæ* Flexner.	2	0	0	0	0
" " Shiga.	3	0	0	0	0
" *friedländeri.*	1	0	0	0	0
" *lactis aerogenes.*	2	0	0	0	0
" *alkaligenes.*	1	0	0	0	0
" *pyocyaneus.*	3	0	0	0	0
" *proteus vulgaris.*	1	0	0	0	0
" *cereus.*	1	0	0	0	0
" *subtilis.*	1	0	0	0	0
" *anthracoides.*	1	0	0	0	0
" *violaceus.*	1	0	0	0	0
Spirillum choleræ asiaticæ.	1	0	0	0	0

fresh guinea pig serum. The work represented in the tables was done as a series of complete experiments. A sample of serum and indicator which was to be employed for the experiment was tested prior to its use to establish its suitability as a substrate for oxidase formation by growing pneumococci.

1. Miscellaneous Group.—Table II represents the results of the study of twenty different microbic species comprising 57 strains.[15] It is at once apparent that, under the same conditions in which the pneumococcus causes complete oxidation of the aminoleucomalachite green, no oxidation of the indicator occurs with the other members of the microbic species tested.

TABLE III.

Positive Oxidase Reaction.
Pneumococcus of Human Origin.

Pneumococcus.	10 per cent horse serum.		10 per cent horse serum with 1 per cent dextrose.		10 per cent fresh guinea pig serum.	Plain broth.	1 per cent dextrose broth.
	Period of observation.						
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.	72 hrs.
F 55	4	4	4	4	4	0	0
A 66	4	4	4	4	4	0	0
J 36	4	4	4	4	4	0	0
F 104	2	4	2	4	4	0	0
Lister G	2	4	2	4	4	0	0
Ca 81	4	4	4	4	4	0	0
Lister F	4	4	4	4	4	0	0
Melford	Tr.	4	2	4	4	0	0
V 1 16	4	4	4	4	4	0	0
Neufeld	4	4	4	4	4	0	0
169	Tr.	4	1	4	4	0	0
168	4	4	4	4	4	0	0
D 40	4	4	4	4	4	0	0
F 208	3	4	3	4	4	0	0
Jones	2	4	2	4	4	0	0
Lister A	2	4	3	4	4	0	0
"	Tr.	4	3	4	3	0	0

2. Pneumococcus.—As with the other bacteria, these strains were picked from single colonies and grown on serum broth and were stained to determine purity prior to use in the test.[16]

[15] With the exception of the meningococcus, one strain each of the organisms given in the table were old stock cultures kindly furnished by Dr. Stanhope Bayne-Jones. The meningococci were stock strains from The Rockefeller Institute. The remaining cultures had been recently isolated from different sources.

[16] We are indebted to Dr. O. T. Avery for the excellent collection of strains of both pneumococci and streptococci here studied. The latter organisms comprised a large portion of those described by Avery and Cullen (Avery, O. T., and Cullen, G. E., The use of the final hydrogen ion concentration in differentiation of *Streptococcus hæmolyticus* of human and bovine types, *J. Exp. Med.*, 1919, xxix, 215).

In contradistinction to the results obtained with the organisms of the above miscellaneous group, nine out of seventeen strains of pneumococci (Table III), irrespective of type, grown on 10 per cent horse serum, with or without dextrose, completely oxidize the leuco compound in a 24 hour period, all of them in 72 hours. The difference between the influence of horse serum and guinea pig serum is here emphasized. Whereas in 24 hours on the horse serum, nine out of

TABLE IV.

Positive Oxidase Reaction.
Streptococcus viridans of Human Origin.

Streptococcus viridans.	10 per cent horse serum pH 7.8.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum pH 7.8.	
	Period of observation.					
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.	72 hrs.
B/26	Tr.	4	0	4	2	4
A/148	"	4	0	4	4	4
A/179	"	4	1	4	4	4
A/141	"	4	0	4	3	4
A/135	"	4	Tr.	4	4	4
380	"	4	"	4	4	4
A/4	"	4	"	4	4	4
R/0	"	4	"	4	4	4
B/38	"	4	"	4	2	4
B/39	1	4	1	4	2	4
B/26	1	4	1	4	4	4

seventeen strains produce a No. 4 reaction (100 per cent), in this same length of time with guinea pig serum, complete oxidation occurred in sixteen out of seventeen, the remaining one producing a reaction but slightly less (No. 3, or 75 per cent).

3. Streptococcus viridans.—*Streptococcus viridans*, like pneumococci, gave a positive reaction with this indicator, but seemingly at a lower rate (Table IV). Growth for 24 hours is marked by only a slight oxidation, which, however, becomes complete in 72 hours. Again, guinea pig serum enhances the reaction, and to such a degree that in the serum of this animal the demarcation between the strains of *Streptococcus viridans* and pneumococci becomes greatly obscured.

TABLE V.

Positive Oxidase Reaction.
Hemolytic Streptococci of Human Origin.

Streptococcus.	10 per cent horse serum pH 7.8, without dextrose.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum pH 7.8.
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
79	Tr.	1	Tr.	4	1
47	"	3	"	4	1
267	0	1	0	2	1
E 7	0	2	0	4	1
43	0	2	0	4	2
A 49	0	4	0	4	3
E 208	0	1	0	4	1
E 223	0	1	0	4	1
239	0	1	0	4	3
E 200	0	2	0	4	2
151	0	1	0	4	1
95	0	0	0	0	1
66	0	2	0	4	1
38	2	2	2	4	2
10	0	2	0	4	2
Boston	0	1	0	4	2
149	0	1	0	4	1
46	0	2	0	4	1
56	0	2	0	4	1
E 238	0	2	0	4	1
8	0	2	0	4	1
27	0	1	0	4	1
92	0	2	0	4	1
89	Tr.	3	1	4	1
25	0	1	0	4	1
S 4	Tr.	2	Tr.	4	1
E 232	0	2	0	2	2
M 29	0	2	0	4	1
M 48	0	1	0	4	1
143	0	0	0	1	3
16	0	1	0	4	1
S 84	0	1	0	4	2
134	0	0	0	0	1
286	0	2	0	4	1
148	0	0	0	4	2
83	0	1	0	4	1
11	0	1	0	4	1
50	0	2	0	4	3
E 265	0	1	0	4	1

TABLE V—*Concluded.*

Streptococcus.	10 per cent horse serum pH 7.8, without dextrose.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum pH 7.8.
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
72	0	2	0	4	1
E 256	0	2	0	4	2
271	0	2	0	2	1
134	0	2	0	4	2
59	0	2	0	2	1
44	0	2	0	4	1
C 67	0	2	0	4	1
2	Tr.	2	0	4	1
39	0	0	0	0	1
15	Tr.	1	0	4	2
140	0	1	0	4	2
48	0	1	0	4	1
C 251	Tr.	Tr.	0	1	1
29	0	1	0	4	1
144	0	1	0	4	2
150	0	3	0	4	1
S 6	0	3	0	4	2
S 71	0	3	0	4	3
S 93	0	3	0	4	Tr.
S 128	0	1	0	4	1
S 116	0	Tr.	0	4	1
S 97	0	1	0	4	1
S 85	0	2	0	4	1
S 35	Tr.	1	0	4	1
S 14	"	1	0	4	2
118	"	1	0	4	1
S 24	0	2	0	4	2
S 1	0	0	0	4	1
E 236	0	2	0	4	1
S 108	0	2	0	4	1
96	0	2	0	4	1
S 148	0	Tr.	0	4	1
209	Tr.	1	0	4	2
F 1	0	Tr.	0	4	1

4. Hemolytic Streptococci of Human Origin.—The study by this test of 73 strains of hemolytic streptococci of human origin demonstrated a difference from pneumococcus and perhaps in a slight degree from *Streptococcus viridans*; that is, with horse serum media, with or

without dextrose, the reaction was negative in a 24 hour period save in the case of one strain (No. 38); on horse serum without dextrose, the reaction after 72 hours ranged from nil with the majority of strains to a complete oxidation with one (No. A 49). But in horse serum to which dextrose had been added, all but ten strains (Nos. 267, 95, E 232, 134, 39, 59, 271, 143, and C 251, Table V) gave a No. 4 (100 per cent) reaction in 72 hours. *A priori* it seems likely that the influence of the dextrose with the serum is one of H ion concentration. That may be true with the hemolytic streptococci of human origin, but as shown above in the miscellaneous group (Table II), in spite of the low final H ion concentration of the media to which dextrose had been added, no oxidation of the indicator resulted. Although H ion concentration influences the rate of oxidation of *p*-aminoleucomalachite green by certain species of organisms that possess this oxidative faculty, in no case in which the reaction was negative for 72 hours was it possible to bring about the oxidation of the indicator by changing the H ion concentration of the serum. The specific oxidizing power seems to be an inherent characteristic of certain species of organisms. The degree of oxidation in guinea pig serum indicates that the hemolytic streptococcus of human origin possesses lower oxidizing activity than either *Streptococcus viridans* or pneumococcus.

5. Hemolytic Streptococci of Milk Origin.—With this group of streptococci the reaction is much less pronounced than with the bacteria studied above (Paragraphs 2 to 4) and the type of reaction approaches closely that of miscellaneous Group 1. All, excepting four strains, reacted either negatively or but slightly positively. One of these (No. M 53) was isolated by Jones[17] from the milk of a cow showing no evidence of mastitis and was considered by him as perhaps of human origin. The uniformity of the reaction with guinea pig serum demonstrates clearly that while these strains are able to oxidize the indicator, the reaction is less pronounced than with the other species studied (Table VI).

6. Hemolytic Streptococci of Cheese Origin.—It is interesting and perhaps significant that the members of this group react in a fashion

[17] Jones, F. S., Studies in bovine mastitis. II. The relation of hemolytic streptococci to udder infections, *J. Exp. Med.*, 1918, xxviii, 253.

TABLE VI.

Variations in the Oxidase Reaction.
Hemolytic Streptococci from Milk.

Streptococci from milk.	10 per cent horse serum pH 7.8.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum pH 7.8.
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
Indian Head.	0	0	0	0	0
Eagle Lunch.	Tr.	0	1	1	3
Munster Brand.	"	0	0	1	4
Philadelphia No. 1.	0	0	0	Tr.	1
" Cream.	Tr.	0	0	0	Tr.
Walker-Gordon.	0	0	0	1	1
Ancre Brand.	0	0	0	0	2
Philadelphia 0.	0	0	0	0	1
Eagle Brand.	0	0	0	0	2
Sheffield (C).	0	0	0	0	3
" (F).	Tr.	Tr.	0	1	1
Crawford Farms.	0	0	0	0	3
Sheffield (E).	0	1	0	4	3
M 3	0	0	0	0	2
M 26	0	2	0	4	2
M 65	0	2	0	2	1
M 43	1	Tr.	1	0	2
M 95	Tr.	0	0	1	1
M 53	0	4	0	4	2

TABLE VII.

Variations in the Oxidase Reaction.
Hemolytic Streptococci from Cheese.

Streptococci from cheese.	10 per cent horse serum pH 7.8.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum pH 7.8.
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
Ch 3	Tr.	0	0	1	3
Ch 12	"	0	1	0	3
Ch 2	"	0	Tr.	Tr.	3
Ch 14	"	0	0	0	3
Ch 10	"	0	0	1	2
Ch 15	0	0	0	0	3
Ch 22	0	0	0	0	1
Ch 11	0	0	0	0	3

similar to those of milk origin. The limited number of strains studied does not warrant the conclusion that the strains from milk and cheese origin can be characterized as identical. However, the streptococci from these sources are differentiated in the present study both from streptococci of human origin and streptococci of udder of cow and mastitis (Table VII).

TABLE VIII.

Variations in the Oxidase Reaction.
Hemolytic Streptococci from Cow's Udder and Mastitis.

Organism.	10 per cent horse serum pH 7.8.		10 per cent horse serum pH 7.8, with 1 per cent dextrose.		10 per cent guinea pig serum.
	Period of observation.				
	24 hrs.	72 hrs.	24 hrs.	72 hrs.	24 hrs.
Streptococci from udder.					
V 3	Tr.	1	0	4	1
V 7 5/6	0	2	0	4	1
V 9	1	4	0	4	3
V 5	0	2	0	4	2
V 2	0	2	0	4	2
V 1	0	2	0	3	1
V 8	0	2	0	4	1
V 20	0	0	0	4	1
Streptococci from mastitis.					
C 69	0	0	0	4	2
C 53	0	2	0	4	1
C 57	0	2	0	4	1
C 67	0	2	0	4	1
C 42	0	2	0	4	1

7. *Hemolytic Streptococci from Udder and Mastitis.*—Whatever the truth concerning the origin of the hemolytic streptococci isolated from milk and cheese, the test is not differential for organisms from human or bovine sources. Strains obtained from the udder or from mastitis of cows give manifestly the same reaction as those of human origin (Table VIII).

DISCUSSION.

It has been shown that certain bacterial species in common with both plant and animal tissue are capable of producing an oxidase as

determined by the oxidation of *p*-aminoleucomalachite green. We are not prepared to state that the effect on this indicator of pneumococci is characteristically that of an oxidase. However, if we hold to Portier's definition of an oxidase,—an enzyme capable of changing gaseous oxygen into active oxygen,—we may assume that the oxidation of *p*-aminoleucomalachite green under the conditions reported in this paper is an indication of the presence of such an enzyme.

In the present report the attempt at the quantitative estimation of the degree of oxidase activity, as judged by the intensity of the color of the oxidized leuco base, has been essentially an approximation. However, sufficient work has since been done with a quantitative colorimeter technique to establish this approximation as a fair index of oxidase activity. In addition, despite considerable difficulty with electrodes, the potential in a typical oxidation and reduction set-up has revealed the possibility of using the method in a more exact study. The feasibility of the measurement of oxidation-reduction potential in biological fluids has been shown by Potter[18] in the case of viable yeast, and Gillespie[19] in the case of bacteria in water-logged soils. More recently, Clark[20] states that he has confirmed Gillespie's work in the main and has been able to develop a system of oxidation and reduction indicators which may be used colorimetrically to determine oxidation-reduction potentials.

The indicator described here separates a relatively small group of microbic species into a group distinct from other known pathogens. The line of demarcation is definite; that is, under the most favorable conditions found by us under which oxidation of the indicator by pneumococci occurs (dextrose in serum) there is not the slightest trace of oxidation discernible with any of the strains studied, except the pneumococcus, *Streptococcus viridans*, and *Streptococcus hæmolyticus*. It is not believed that the other bacteria are incapable of oxidation of this character, but rather that the oxidation potential produced by the

[18] Potter, M. C., Rate of fermentation as measured by difference of potential, *Proc. Univ. Durham Phil. Soc.*, 1912, iv, 230.

[19] Gillespie, L. J., Reduction potentials of bacterial cultures and of water-logged soils, *Soil Science*, 1920, ix, 199.

[20] Clark, W. M., Studies on oxidation-reduction. I. Introduction, *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 443.

growing organism is not effective for the indicator in question. This view is strengthened by the fact that there is considerable variation in the degree of oxidation among the species giving a positive reaction with this leuco base. Pneumococcus gives the most intense reaction, *Streptococcus viridans* next, and *Streptococcus hæmolyticus* follows, the result varying in part with the source of the organism. The findings with the hemolytic streptococci of human origin and cow's udder and mastitis are not only most pronounced relatively speaking but are indistinguishable from one another, while the organisms from milk and cheese though apparently similar to those just mentioned are of lower oxidative power. We consider these results as a preliminary study. Further work has been confirmatory, but an insufficient number of strains of organisms has been studied to make generalization advisable.

SUMMARY AND CONCLUSIONS.

1. A simple technique is described for studying the oxidase action of bacteria by means of the oxidation of *p*-aminoleucomalachite green.

2. It is shown that pneumococci under aerobic conditions produced an oxidase when grown on suitable medium. The sera of any of seven different animal species constitute such a medium, the degree of oxidation by the pneumococcus depending upon the animal from which the serum was taken—rat, guinea pig, rabbit, horse, man, cat, and chicken in order of diminishing suitability.

3. Conditions favoring the oxidation of *p*-aminoleucomalachite green by a single strain of pneumococci are: the presence of a slight amount of hemoglobin, dextrose, H ion concentration on the acid side, and heating of fresh serum for 30 minutes at 56°C. Conditions preventing the oxidation are: sterilized meat infusion, 1 per cent peptone, plain broth, a high concentration of hemoglobin, and absence of oxygen. In a quantitative fashion, meat infusion, 1 per cent peptone, and plain broth interfere with the suitability of serum as a substratum of oxidase production by the pneumococcus.

4. Twenty-three microbic species were studied with reference to oxidative power. They were grown upon 10 per cent horse serum, with and without dextrose, upon 10 per cent guinea pig serum, and upon plain broth. Only three of the twenty-three gave evidence of oxida-

tive power as tested by *p*-aminoleucomalachite green; namely, the pneumococcus, *Streptococcus viridans*, and *Streptococcus hæmolyticus*. Among the strains, of these three pneumococci gave the most intense reaction, after which *Streptococcus viridans* and *Streptococcus hæmolyticus* follow in the order named, but with a noticeable variation among the different strains of *Streptococcus hæmolyticus*.

5. Hemolytic streptococci of human and bovine origin were studied. The only variation in the type of reaction was manifested by the streptococci of milk and cheese origin. Strains from these sources showed definitely the least oxidase activity. Streptococci from mastitis and cow's udder were indistinguishable by the test from the hemolytic streptococci of human origin.

STUDIES ON X-RAY EFFECTS.

XIII. Histological Study of the Fate of Cancer Grafts Inoculated into an X-Rayed Area.

By WARO NAKAHARA, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 22 and 23.

(Received for publication, May 28, 1923.)

The familiar phases of cancer cell degeneration following x-ray treatment in man have generally been attributed to the direct injury produced by the irradiation, but there is little experimental proof to support this idea. On the contrary, recent studies have indicated that the treatment dose of x-rays has little, if any, direct effect on the cancer cells, and that the curative action of this agent depends largely, if not entirely, on the reaction induced in the surrounding normal tissue.[1] The basis for this conclusion is to be found in the fact that a cancer graft will rarely grow when inoculated into a region which has previously been exposed to an erythema dose of x-rays, while grafts of the same tumor grow actively in unradiated parts of the same animal.

Under the conditions of an experiment such as that described above, the cancer cells inoculated into an x-rayed area themselves received no x-rays, and therefore any changes taking place in them must be secondary to the altered condition induced by x-rays in the surrounding tissue. The object of the present investigation is to examine histologically the series of degenerative changes taking place in cancer cells implanted in a location made unfavorable for their growth by a previous exposure to x-rays.

[1] Murphy, Jas. B., Hussey, R. G., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 299.

EXPERIMENTS.

Sixteen mice, after etherization, were shaved in the upper abdominal region down to and including both groins, and were then secured on a small board. An opening, measuring about 15 by 20 mm., was cut out of a piece of sheet lead, and this was placed over the animal so as to expose the left groin, to the midline, and completely protect the rest of the body. This area was then given a dose of x-rays governed by the following factors: 3 inch spark-gap, 10 milliamperes, 6 inch distance from the target, and time of exposure 2½ minutes.

7 days later, when a mild erythema was first appearing, cancer grafts (Bashford Adenocarcinoma No. 63) were inoculated intracutaneously in the x-rayed area and like grafts in the corresponding location of the right, non-radiated groin.

Six of the sixteen mice were killed, a pair at a time, 48 hours, 4 days, and 7 days after the cancer inoculation. The cancer grafts with the surrounding tissue in the x-rayed and protected groins were removed and fixed in Carnoy's chloroform-alcohol-acetic fluid. The remaining ten mice were killed in pairs 24 hours, 48 hours, 3 days, 5 days, and 7 days after cancer inoculation, and the grafts were fixed in formaldehyde. Sections were stained with methylene blue or hematoxylin and eosin.

In order to determine what would have been the outcome of the tumor inoculations, six additional mice were subjected to the same x-ray treatment and inoculations, but were allowed to live. In the course of 3 weeks five out of six inoculations in the protected area resulted in healthy tumors, while only one tumor developed out of six inoculations made in the x-rayed area.

Histological Description.

The grafts removed 24 hours after inoculation were found to consist of a necrotic mass with islands of healthy cancer cells scattered here and there. In and around the grafts there was an acute polymorphonuclear and a mild lymphoid reaction. In the condition of the inoculated material and the reaction immediately surrounding it, no difference could be discovered between the grafts from the

two sides, but in the x-rayed area there was a marked lymphoid reaction in the skin layers, which, however, did not come in direct contact with the graft.

In the x-rayed area the necrotic debris of the original mass of graft tissue had been largely removed at the end of the 48 hour and 3 day periods, and the islands of healthy looking cancer cells showed frequent mitotic figures and seemed larger than at the previous period. These islands often formed a more or less continuous ring around the remains of the necrotic mass. The polymorphonuclear reaction had almost subsided and was replaced by a more extensive reaction of the cells of the lymphoid variety (Fig. 1). There was also pronounced activity of the fibroblastic tissue, and the cellular infiltration of the skin layers was still very prominent. At the same period cancer grafts in the protected areas were found to be in a more active stage of growth, islands of cancer cells tending to coalesce and to form irregularly shaped masses of healthy looking tissue. The polymorphonuclear reaction was much reduced, as in the x-rayed area, while the lymphoid cell infiltration was now present to only a limited extent around the cancer grafts.

At the 4 to 5 day periods the difference between the cancer grafts in x-rayed areas and those in protected areas was that degenerative changes had become even more prominent in the former, while the latter continued to grow actively.

The first event in this degenerative process, as seen under the microscope, was the swelling of both nucleus and cytoplasm. The cytoplasm gradually became more acidophilic, and the nucleus hyperchromatic (Fig. 2). The nucleus finally lost its structure, became more deeply stained, and later uniformly pycnotic. The general cell structure also became less and less distinct, and with the fragmentation of the pycnotic nuclei the cells were reduced to debris.

It was observed also that two or more cells often coalesced to form giant cells with highly vacuolated cytoplasm (Figs. 3 and 4), this type of change being found more frequently in small groups which had become imbedded in a fibrous matrix (Figs. 3, *A* and 4, *B*). It was not uncommon to find the nuclei pushed to the periphery of some cells by large masses of inclusion bodies (Fig. 3, *B*). In later stages of degeneration the nuclei lost their staining capacity and

appeared as the so called "ghost nuclei" (Fig. 4, *B*), and then finally became unrecognizable, leaving the cells as irregularly shaped masses of homogeneous appearance.

This degeneration process was generally complete by the 7th day, when the remains of the graft in the x-rayed area were represented by a small mass of necrotic debris, attended by some polymorphonuclear cells and macrophage reaction.

DISCUSSION.

It may be noted that the series of changes described above are in every respect similar to those that have been reported by Apolant,[2] Contamin,[3] Clunet,[4] Marie and Clunet,[5] Colwell and Russ,[6] Knox,[7] and others, as the typical changes taking place in cancer cells following radiation. To summarize the statements of these authors, all of whom describe essentially the same series of events, the first change occurring after the irradiation of cancer is a lymphocytic and to a less extent a polymorphonuclear exudation, and a connective tissue proliferation around the neoplasm. A few days later the cancer cells themselves begin to show signs of degeneration. These degenerative changes entail a marked swelling of both nucleus and cytoplasm, loss of structural details, hyperchromatism, pycnosis, and finally fragmentation of the nucleus, appearance of vacuoles, with increase in acidophilic affinity of cytoplasm, and giant cell formation by cell fusion. As the lymphocytes and connective tissue stroma increase, the cancer tissue is broken up into small islands, and at the final stage the pycnotic nuclei disintegrate and all traces of living cells are lost.

[2] Apolant, H., *Deutsch. med. Woch.*, 1904, xxx, 454.

[3] Contamin, M. A., *Compt. rend. Acad.*, 1910, cl, 1537.

[4] Clunet, J., cited by Woglom, W. H., The study of experimental cancer, a review, New York, 1913.

[5] Marie, P. L., and Clunet, J., cited by Woglom, W. H., The study of experimental cancer, a review, New York, 1913.

[6] Colwell, H. A., and Russ, S., Radium, x-rays and living cells, London, 1915.

[7] Knox, R., Radiography, x-ray therapeutics, and radium therapy, New York, 1916.

The general deduction has been that these characteristic changes resulted from the direct injury inflicted on the cancer cells by the x-rays. That this deduction is open to question is shown by the facts presented here; namely, that cancer cells implanted in an area of skin previously exposed to x-rays undergo exactly the same series of degenerative changes as those supposed to result from the direct injury.

In the case of radium treatment, especially when the emanation tube is imbedded in cancer tissue, there is little doubt that some of the cancer cells are directly killed by this agent. Recent observations by Ewing[8] and Alter[9] suggest, however, that the direct injury alone can hardly account for the entire process, and, furthermore, the effect of imbedded emanation tube on normal tissue as reported by Bagg[10] indicates that the local accumulation of lymphocytes, rather than the necrosis of tissues immediately surrounding the tube, may be the more extensive of the induced changes. It would seem probable, therefore, that with radium as well as with x-rays, the induced reaction in the surrounding tissue plays the important rôle in bringing about the destruction of the cancer cells.

SUMMARY.

Cancer cells implanted in a skin region previously exposed to an erythema dose of x-rays show a series of degenerative changes in every way comparable to the frequently described stages of cancer cell degeneration following x-ray treatment. The findings contrast strongly with the survival and growth of grafts implanted in unexposed regions in the same animal. Since the changes are the same whether the cancer cells have been directly exposed *in situ* or merely implanted in the previously exposed skin, it follows that it is impossible to establish microscopically a direct injury from the x-raying as the principal factor in the therapeutic action of x-rays on cancer.

[8] Ewing, J., *J. Am. Med. Assn.*, 1917, lxviii, 1238.
[9] Alter, N. M., *J. Med. Research*, 1919, xl, 241.
[10] Bagg, H. J., *Am. J. Roentgenol.*, 1920, vii, 536.

EXPLANATION OF PLATES.

PLATE 22.

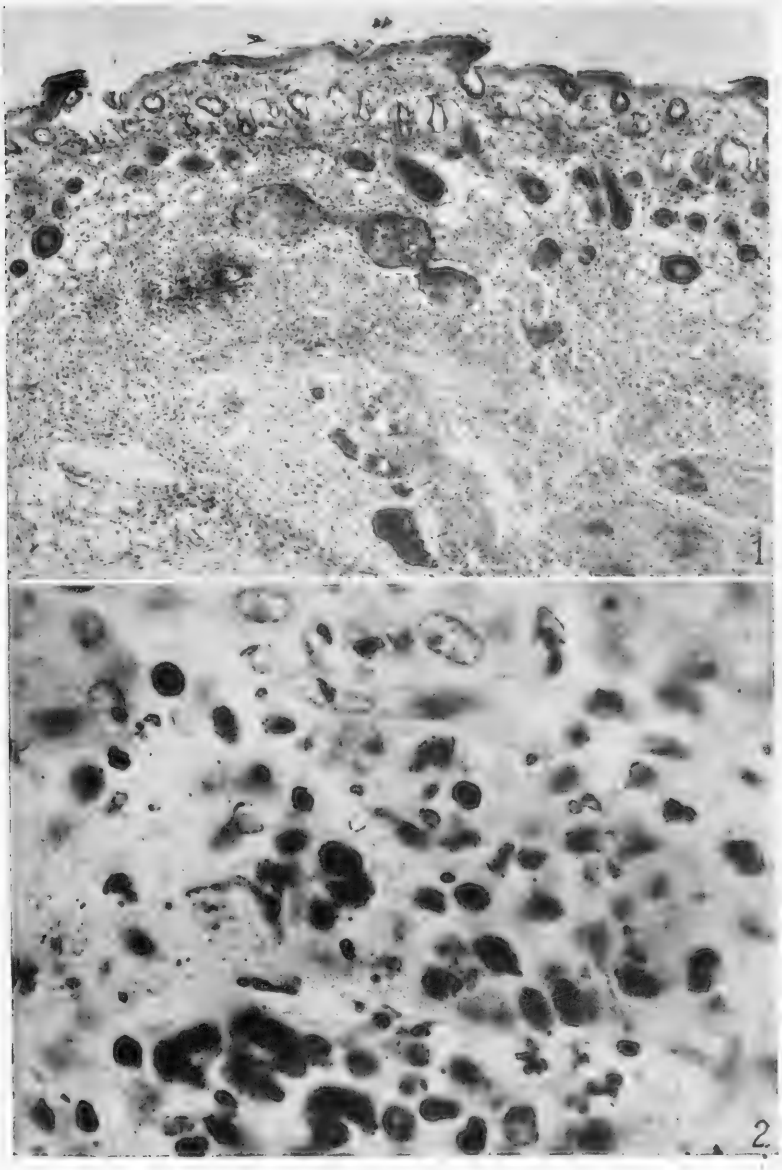
FIG. 1. 48 hour cancer graft in the area of skin exposed to an erythema dose of x-rays 7 days previous to cancer inoculation. Small islands of cancer cells are shown in the midst of the skin layers thickly infiltrated, principally by cells of the lymphoid variety.

FIG. 2. Part of a 5 day graft in x-rayed skin. Hyperchromatism, pycnosis, and fragmentation of nuclei are represented. Some healthy living cancer cells are shown at the top.

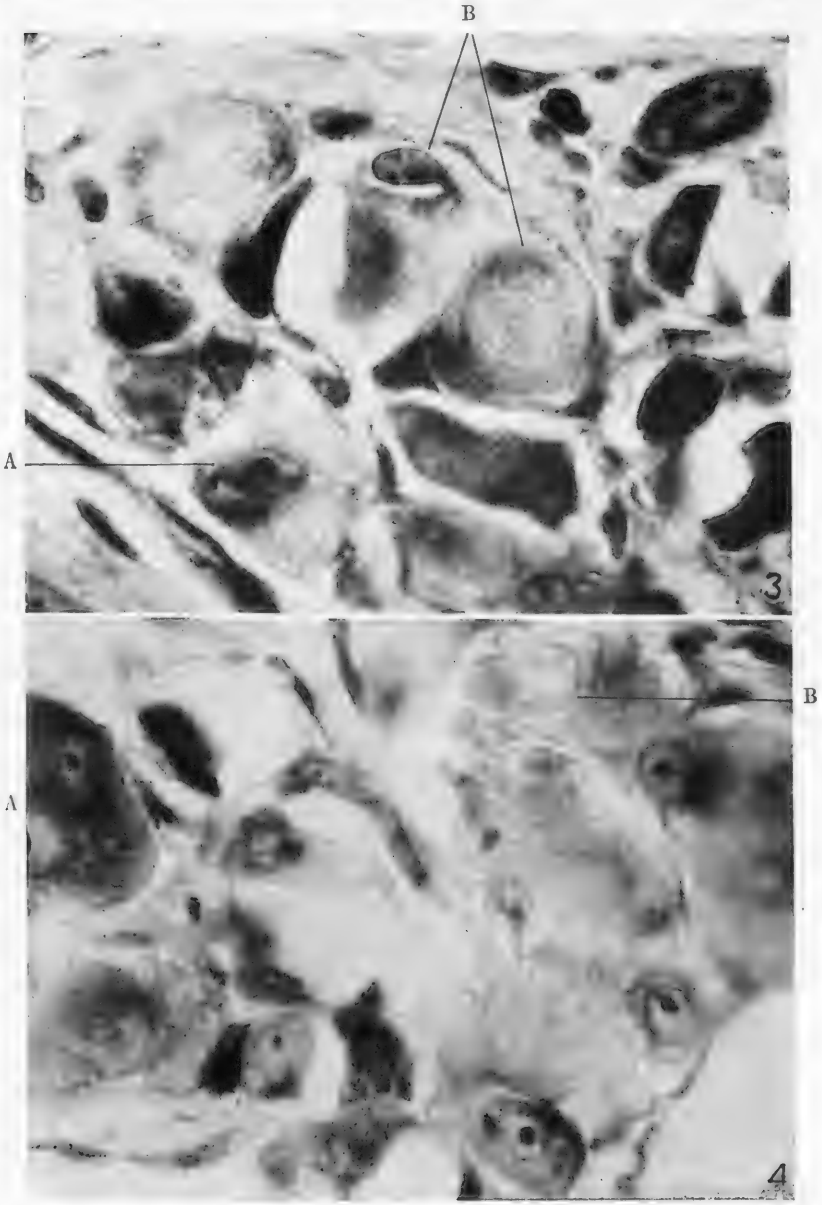
PLATE 23.

FIG. 3. A small group of cancer cells imbedded in fibrous tissue, showing binucleated and highly vacuolated giant cell (*A*) and giant cells with nuclei pushed to the periphery (*B*).

FIG. 4. The same as Fig. 3, showing a binucleated giant cell (*A*) and so called "ghost cells" (*B*).



(Nakahara: Studies on x-ray effects. XIII.)



(Nakahara: Studies on x-ray effects. XIII.)

STUDIES ON LYMPHOID ACTIVITY.

VII. Suppression of Induced Immunity to Transplanted Cancer by Large Doses of Olive Oil.

By WARO NAKAHARA, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 7, 1923.)

As previously reported, a small quantity of olive oil injected intraperitoneally in mice, induces a typical form of immunity to transplantable cancer.[1] Incidentally, it was noted that olive oil, while in small doses increases resistance, in sufficiently large doses renders mice hypersusceptible to the growth of cancer transplants. This quantitative relation presents an interesting parallel to the action of x-rays, with which agent, by changing the dosage properly,[2–4] the resistance of mice to transplantable cancer can almost at will be either increased or decreased.

An artificial immunity can also be broken down by means of comparatively massive doses of x-rays.[3,5,6] The present study was undertaken to determine whether or not olive oil also is capable of setting aside the state of so called induced immunity to transplanted cancer.

Effect of Olive Oil Injected into Immunized Mice Immediately before Cancer Inoculation.

In all the experiments to be reported here, commercial olive oil, described as the first expression, was used. Injections were made

[1] Nakahara, W., *J. Exp. Med.*, 1922, xxxv, 493.

[2] Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.

[3] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

[4] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429; 1922, xxxv, 475.

[5] Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.

[6] Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917–19, xc, 1.

intraperitoneally in amounts of 0.7 cc. each, which dose was found adequate, although a larger quantity, even up to 1 cc., can be safely administered in a single injection. The mice for the experiments were young adults from one stock, and all of about the same size. Bashford Adenocarcinoma No. 63 was employed for transplantations.

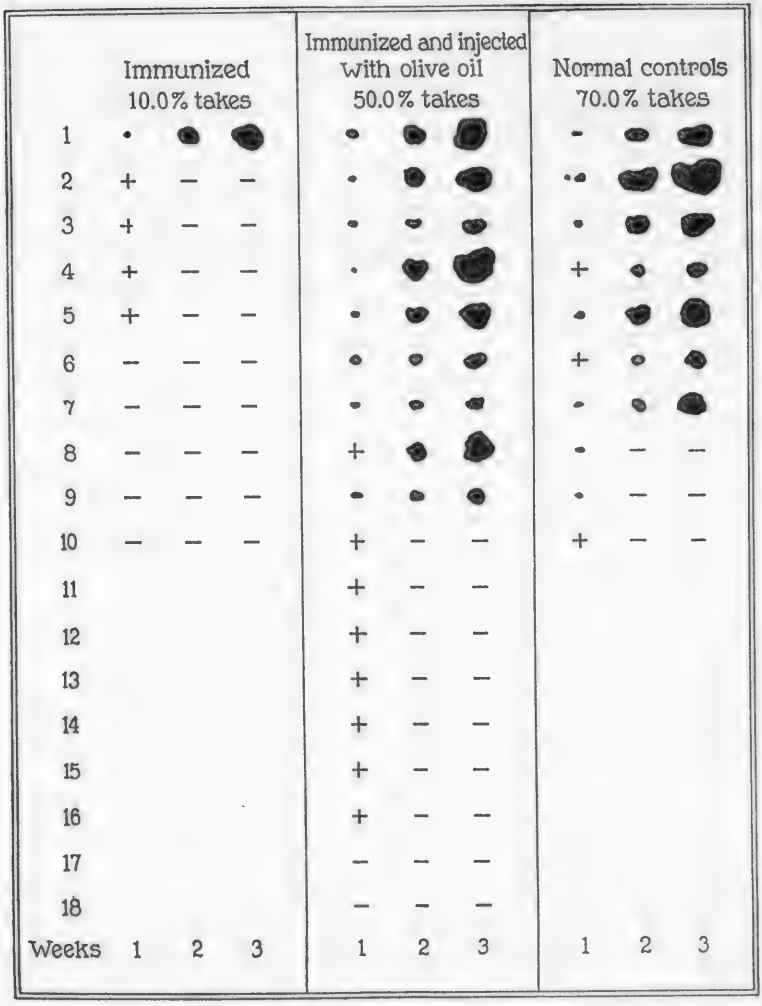
Experiments 1 to 4.—An immunizing injection of 0.2 cc. of defibrinated mouse blood was given to a number of mice which were divided into two groups. 10 days later those of one group were injected with olive oil, and immediately afterward inoculated with bits of the Bashford tumor. The other group, and a suitable number of normal, untreated mice, were inoculated with the tumor at the same time (Table I).

TABLE I.

Experiments 1 to 4.

Experiment No.	Takes.		
	Group 1. Immunized.	Group 2. Immunized and oil-injected.	Group 3. Controls.
1	22.2 per cent (9 mice).	71.5 per cent (7 mice).	100.0 per cent (9 mice).
2 (Text-fig. 1).	10.0 per cent (10 mice).	50.0 per cent (18 mice).	70.0 per cent (10 mice).
3	0.0 per cent (9 mice).	35.3 per cent (17 mice).	44.4 per cent (9 mice).
4	25.0 per cent (8 mice).	65.0 per cent (20 mice).	
Average.....	13.9 per cent (36 mice).	53.2 per cent (62 mice).	71.4 per cent (28 mice).

The results show conclusively that by injecting a large quantity of olive oil in potentially immune mice, immediately before cancer inoculation, the number of takes in these mice can be so increased as to approach that in normal controls. The percentage of takes was far greater than in mice immunized but given no olive oil.


Immunized
10.0% takes
Immunized and injected with olive oil
50.0% takes
Normal controls
70.0% takes
1 • ● ● ● ● ● - ● ●
2 + – – • ● ● ·● ● ●
3 + – – • ● ● • ● ●
4 + – – • ● ● + ● ●
5 + – – • ● ● • ● ●
6 – – – ● ● ● + ● ●
7 – – – • ● ● • ● ●
8 – – – + ● ● • – –
9 – – – • ● ● • – –
10 – – – + – – + – –
11 + – –
12 + – –
13 + – –
14 + – –
15 + – –
16 + – –
17 – – –
18 – – –
Weeks 1 2 3 1 2 3 1 2 3

TEXT-FIG. 1. Relative sizes of tumors in Experiment 2. Cancer inoculation was made in the immunized group 10 days after immunization; in the immunized and oil-injected group 10 days after immunization and immediately after olive oil injection.

Duration of the Suppressive Effect of Olive Oil on Potential Immunity.

The next experiments dealt with the duration of the suppressive effect of the olive oil injection.

Experiments 5 to 7.—Mice were immunized and divided into two groups as in preceding experiments. 10 days later one group was injected with olive oil, while the other group received no injection. 17 days after the immunizing blood injection, and 7 days after olive oil injection, all the mice were given tumor grafts, as were a number of normal mice. The results are shown in Table II.

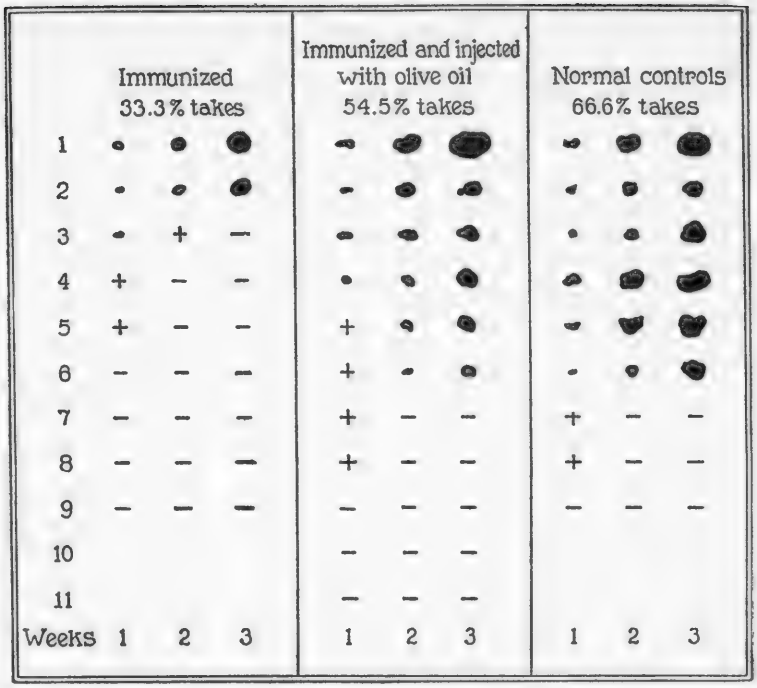
TABLE II.

Experiments 5 to 7.

Experiment No.	Takes.		
	Group 1. Immunized.	Group 2. Immunized and oil-injected.	Group 3. Controls.
5 (Text-fig. 2).	33.3 per cent (9 mice).	54.5 per cent (11 mice).	66.6 per cent (9 mice).
6	40.0 per cent (10 mice).	70.0 per cent (10 mice).	75.0 per cent (8 mice).
7	30.0 per cent (10 mice).	55.5 per cent (9 mice).	70.0 per cent (10 mice).
Average.....	34.5 per cent (29 mice).	60.0 per cent (30 mice).	70.3 per cent (27 mice).

It is evident from the above figures that the depressive effect of olive oil on the resistance is still operative 7 days after the injection. Although there is some falling off in the general effect of the induced resistance, there is still considerable difference between the number of takes in the immunized group which was given oil and the one which was not. As the duration of the induced immune state is limited to a few weeks,[7] it was considered unprofitable to make a test at a later period.

[7] Bashford, E. F., *Brit. Med. J.*, 1906, ii, 209. Bashford, E. F., Murray, J. A., and Cramer, W., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 164. Woglom, W. H., *J. Exp. Med.*, 1912, xvi, 629.



TEXT-FIG. 2. Relative sizes of tumors in Experiment 5. Cancer inoculation was made in the immunized group 17 days after immunization; in the immunized and oil-injected group 17 days after immunization and 7 days after oil injection.

Effect of Olive Oil Injected into Immunized Mice after Cancer Inoculation.

Further experiments were undertaken to determine whether the immunity process, already called into action by a cancer inoculation in potentially immune mice, can be influenced by olive oil injection. As the destruction of cancer grafts in immune hosts is often complete in a short time, the oil injections in these experiments were made 24 hours after the cancer grafting.

Experiments 8 and 9.—Mice were immunized by the injection of blood as before, and 10 days later were inoculated with a tumor, together with a suitable number of normal controls. 24 hours after the cancer inoculation half the immunized mice were inoculated with olive oil. Table III shows the effects.

TABLE III.

Experiments 8 and 9.

Experiment No.	Takes.		
	Group 1. Immunized.	Group 2. Immunized and oil-injected.	Group 3. Controls.
8	10.0 per cent (10 mice).	10.0 per cent (10 mice).	70.0 per cent (10 mice).
9	30.0 per cent (10 mice).	20.0 per cent (10 mice).	70.0 per cent (10 mice).
Average.....	20.0 per cent (20 mice).	15.0 per cent (20 mice).	70.0 per cent (20 mice).

The results are of interest in showing that although it may be possible to increase the per cent of takes in immunized mice by administering larger doses of olive oil, the amount sufficient to affect the potential immunity is insufficient to exert any perceptible influence on the active immunity; that is, while it is possible to prevent the development of the resistance mechanism, it is impossible, apparently, at least by these means, to suppress it after it has been activated by cancer inoculation.

Modification of Cellular Reactions Accompanying Immunity.

Characteristic cellular manifestations of three varieties are now known to accompany the resistant state of animals to transplanted cancer: (1) marked lymphocytic infiltration around the inoculated grafts;[1,8] (2) stimulation of cell division in germ centers of lymphoid organs;[9] and (3) hyperlymphocytosis in the blood.[3] It is of interest

[8] Burgess, A. M., *J. Med. Research*, 1909, xxi, 575. Da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1. Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1912, xv, 270. Tyzzer, E. E., *J. Med. Research*, 1915, xxxii, 201.

[9] Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

to ascertain the effect of olive oil injections on these associated cellular reactions in instances in which the dose used was sufficient to suppress the immunity itself.

Experiment 10.—Ten normal white mice were immunized by subcutaneous injection of 0.2 cc. of mouse blood. 10 days later, they were injected intraperitoneally with 0.7 cc. of olive oil and immediately thereafter inoculated with fragments of the Bashford adenocarcinoma. These mice were killed in pairs, at intervals of 24 hours, 48 hours, 3 days, 4 days, and 5 days after the inoculation.

For comparison, ten normal mice were immunized as above, and 10 days later grafts of the tumor were similarly inoculated, but without the preceding injection of olive oil. These mice were killed, two at a time on 5 successive days as in the preceding instance.

The cancer grafts and surrounding tissue, the spleen, and lymph nodes were removed at autopsy, preserved in Carnoy's fluid, and prepared for microscopic study.

Local Lymphoid Infiltration.—The absorption of the exudate resulting from the trauma of inoculation was generally complete within 48 hours, after which only slight evidence of cellular reaction was seen in the tissue surrounding the cancer graft in the oil-injected series. During the early stages there were but few lymphocytes immediately surrounding the cancer graft, and a very slight degree of connective tissue proliferation. Occasionally, however, small groups of lymphocytes were present around blood vessels. Later, the connective tissue stroma became more abundant, the graft received a copious vascular supply, and lymphocytic infiltration also appeared, but only to a slight extent.

Cancer grafts from the series which were immunized and cancer-inoculated, but not given an olive oil injection, offered a marked contrast to the picture described above. There was a dense cellular infiltration of the tissue surrounding the cancer graft which was composed of lymphocytes, plasma cells, and fibroblasts, and in addition a perivascular reaction occurred about small vessels in the loose connective tissue beyond the edge of the main reaction, a typical manifestation of local reaction in cancer immunity.

Lymphoid Organs.—Histological study of the spleen and lymph nodes from the two series of animals showed no definite differences. The general impression derived therefrom was that in the animals

which had received oil, proliferation of the lymphoblasts in the germ centers was less intense than in the animals which had received no oil, but this difference, if present, was slight.

Blood Lymphocytosis.—The following experiments were carried out to determine the effect of the olive oil injection on the blood picture which ordinarily accompanies the immunity process.

Experiment 11 (*Text-Fig. 3*). *Series A.*—Counts were made on eight mice before and at intervals after immunization and cancer implantation, repeating, for the sake of comparison, the original observation of Murphy and Morton.[3] Of these eight mice, all but two proved to be immune to cancer inoculation (Table IV).

TABLE IV.

Lymphocyte Counts on Series A (*Immunized*).

Mouse No.	1 day before blood injection.	3 days after cancer inoculation.	10 days after cancer inoculation.	Outcome of cancer inoculation.
1	9,534	10,445	7,613	+
2	12,364	11,181	9,858	+
Average for susceptible mice....	10,949	10,813	8,735	
3	9,106	16,862	14,703	−
4	6,528	19,759	12,483	−
5	8,827	9,445	14,468	−
6	11,496	16,855	12,695	−
7	10,024	13,344	10,719	−
8	6,287	12,854	15,411	−
Average for immune mice.....	8,711	14,853	13,413	
Average for all the mice.....	9,273	13,843	12,368	75.0 per cent immune.

Series B.—Counts were made on twenty mice immunized and grafted with cancer as in Series A, and in addition injected with 0.7 cc. of olive oil immediately preceding the cancer inoculation. In this series, thirteen out of twenty mice developed tumors (Table V).

Absolute counts of the lymphocytes, including large and small lymphocytes, large mononuclear cells, and transitionals, per cubic millimeter of blood in the above series of mice, are given individually in Tables IV and V, and the averages are charted in Text-fig. 3. Polymorphonuclear counts have not been included because they showed no material variation.

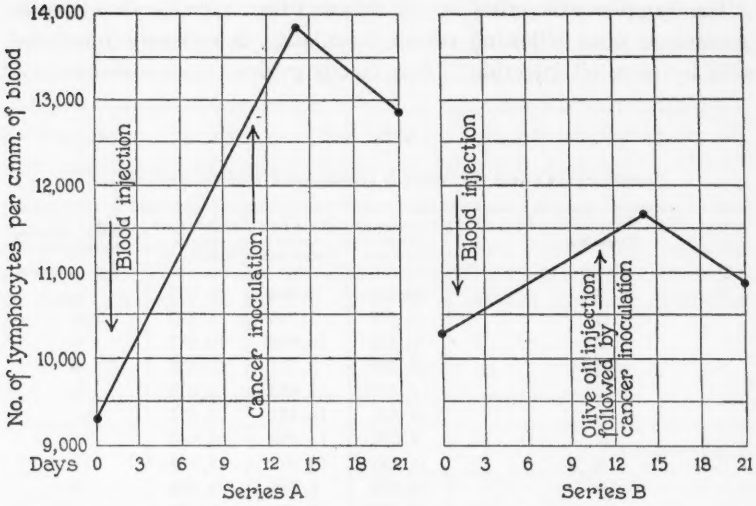
The lymphocytic crisis in the blood which usually develops in immunized mice following cancer inoculation is seriously interfered with by olive oil injection. This fact is evident from a comparison

TABLE V.

Lymphocyte Counts on Series B (Immunized and Oil-Injected).

Mouse No.	1 day before blood injection.	3 days after cancer inoculation.	10 days after cancer inoculation.	Outcome of cancer inoculation.
1	10,473	8,894	8,675	+
2	6,578	7,249	6,555	+
3	10,381	10,903	10,087	+
4	12,276	8,711	8,648	+
5	7,741	7,613	7,076	+
6	10,332	10,451	8,627	+
7	9,778	11,198	9,936	+
8	11,838	13,239	12,282	+
9	10,331	6,986	9,104	+
10	6,980	9,615	9,679	+
11	8,659	6,673	6,128	+
12	13,848	10,539	11,833	+
13	17,042	17,153	10,685	+
Average for susceptible mice....	10,481	9,940	9,178	
14	12,221	21,386	18,195	−
15	13,868	15,733	18,316	−
16	11,026	15,377	16,243	−
17	5,660	13,210	12,506	−
18	7,694	14,358	11,334	−
19	13,442	16,896	12,921	−
20	5,752	8,890	8,812	−
Average for immune mice.....	9,951	15,121	14,046	
Average for all the mice.....	10,296	11,753	10,882	35.0 per cent immune.

of the average variation of lymphocytes of Series A and B (Text-fig. 3). In Series B a small proportion of mice showed a lymphocytosis in spite of olive oil injection, and these mice later proved to be immune to the inoculated cancer.



TEXT-FIG. 3. Experiment 11. Composite curve of the lymphocyte counts on eight mice immunized and inoculated with cancer 10 days later (Series A), and on twenty mice immunized and 10 days later injected with olive oil followed by cancer inoculation (Series B).

Histological Changes in Normal Mice after Injection of a Large Dose of Olive Oil.

With the suppressive action of olive oil on induced cancer immunity and on certain of its associated cellular reactions established, it has become desirable to ascertain the effect of such injections on normal mice. In an earlier communication,[1] a preliminary observation was noted; namely, that intraperitoneal injections of olive oil in large doses suppress the proliferation of lymphoid cells. The object of the next experiment has been primarily to repeat the work with a view to substantiating this point.

Experiment 12.—Ten normal mice were injected with 0.7 cc. of olive oil intraperitoneally, and were killed in pairs 48 hours, and 4, 7, 10, and 14 days after the injection.

Peritoneal Cavity.—It was noted, at autopsy, that the peritoneal cavities of all the mice were filled with unemulsified oil, in which only a very few cells could be seen microscopically.

Spleen.—At the 48 hour period the germ centers were found to be very small and showed an almost complete suppression of mitosis. The Malpighian bodies themselves were also much reduced in size, but there was no unusual amount of necrosis. Such changes could not be detected at the later periods.

Lymph Nodes.—The changes here were much the same, apparently, as those in the spleen; no unusual condition other than a transitory suppression of mitosis in the germ centers was detected.

Suprarenal Glands.—In every mouse killed 48 hours, and 4 days after the injection, some mitotic figures were seen among the cells of the cortex. On the average, one dividing cell was found for every two sections, but occasionally two were seen in the same section. At later periods no mitotic figures were found, and the condition appeared normal.

Other Organs.—Liver, kidney, bone marrow, and thymus and thyroid glands were examined, but showed no noticeable change.

The above findings corroborate the results previously reported as concerns the lymphoid changes, and they add an interesting manifestation in the suprarenal gland. The significance of the latter change is not apparent at present.

DISCUSSION.

The effect of large doses of olive oil on the lymphoid tissue differs from the x-ray effect, in that the former brings about merely a suppression of activity of the cells, while the latter actually destroys them. Yet olive oil is very nearly as potent in its interference with immunity to engrafted cancer as are x-rays.

It has often been suggested that induced resistance to cancer is a phenomenon closely related in type to the anaphylactic reaction. This notion is somewhat strengthened by the fact that x-rays not only destroy resistance to transplanted cancer,[3] but also prevent, as shown by von Heinrich[10] and by Hussey,[11] the sensitization of an

[10] von Heinrich, H., *Centr. Bakt., Ite Abt., Orig.*, 1913, lxx, 421.

[11] Hussey, R. G., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 22.

animal to a foreign protein. Unsaturated fatty acids are likewise capable of interfering with the development of sensitiveness to foreign proteins.[12] The present experiments demonstrate that olive oil, rich in unsaturated fatty acids, is also capable of annulling the immunity reaction to transplanted cancer.

SUMMARY.

The state of potential immunity to transplanted cancer engendered in mice by an injection of homologous blood may be reversed and converted into a susceptible state by an intraperitoneal injection of olive oil prior to the cancer inoculation.

The suppression of resistance is accompanied by the failure of certain cellular reactions known to develop during the establishment of immunity to transplanted cancer.

[12] Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, xx, 468.